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# BACTERIAL STEM AND LEAF ROT OF DIEFFENBACHIA IN FLORIDA

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SUMMARY

A serious disease of dieffenbachia was first recognized in 1956 causing extensive plant losses in southern Florida. The disease is characterized by brownish, watersoaked, sunken, soft areas on the stems, and irregular, brownish, soft spots on the leaves. Favored by high temperature and moisture, the pathogen may attack plants of any age. Studies of its cultural, morphological, and biochemical characteristics, in addition to cross-inoculation tests, indicate the pathogen is a new species; the name **Erwinia dieffenbachiae** sp. n. is proposed.

Partial disease control resulted when inoculated cane sections were immersed in Agri-mycin 100, or hot water. A combination of these treatments also gave satisfactory control.

INTRODUCTION.—A recent paper by Munnecke (14) described a bacterial stem rot disease of dieffenbachia in California which appears similar to 1 causing economic losses of dieffenbachia in Florida. Positive identity of the 2 diseases is not yet known, since Munnecke did not describe the pathogen, but considered it a strain of *Erwina chrysanthemi* Burkh., *et al.* In addition to dieffenbachia stem rot, a leaf spot of dieffenbachia is caused by the same bacterium in southern Florida. This present paper will discuss certain aspects of the disease and pathogen not reported by Munnecke.

In Florida, the disease was first observed in 1956 on plants of *Dieffenbachia picta* var. *amoena* Hort. from Apopka, Fla. During the past 4 years, the disease has been observed on several other *Dieffenbachia* spp. and from several other areas of the state. When first observed, the disease resembled the stem rot disease reported by Tompkins and Tucker in 1947 (16), caused by *Phytophthora palmivora* Butler. Congo Red smear preparations made using diseased tissue, however, showed the presence of bacteria in both stem and leaf lesions. When dilution plates were prepared, the affected tissues yielded several types of bacterial colonies, 1 of which proved highly pathogenic to dieffenbachia, producing symptoms indistinguishable from natural infections.

First reports of disease symptoms and the causal agent were made by McFadden (11, 12, 13). Baker and Chandler (1) in 1956 mentioned a bacterial soft rot disease of dieffenbachia and cited *E. carotovora* (Jones) Holland as the probable cause. A bacterial leaf spot of *D. picta* (Lodd.) Schott caused by *Xanthomonas dieffenbachiae* (McCullock & Pirone) Dowson was described in 1939 (10).

Dieffenbachias, like many tropical foliage plants, are grown for their attractive ornamental foliage. Since infected plants are unsaleable, losses have been extensive, frequently involving 25% or more of a planting and in some instances curtailing expansion of the crop. The insidious nature of the disease, in that plants may be infected yet show no visible symptoms, makes its detection difficult and adds to its importance to nurserymen. Plants may develop the disease at any stage of growth.

*Stem symptoms.*—Stem or cane lesions may occur above or below the soil surface. When below the soil surface, lesions may go unnoticed until the rot becomes advanced. Stem lesions are watersoaked, soft, and grayish at first, later becoming tan to pale brown, sunken, and irregular in shape but with a clear line separating the diseased and healthy tissues (Fig. 1-A, B). Affected plants characteristically produce terminal leaves which are pale yellow and reduced in size. In many instances, this is the only visible aboveground symptom. Under favorable conditions, the stem lesions rapidly advance causing the lower leaves to turn yellow and prematurely die. Frequently, the organism becomes systemic and invades the petioles and midribs (Fig. 1-C). Such areas become watersoaked and brownish in color; the leaves collapse and become soft and mushy.

*Leaf symptoms.*—On *D. picta* var. *amoena* leaf infections are characterized by small pin-point, watersoaked, pale brown spots, measuring 1-2 mm in diameter and surrounded by a diffused yellow margin (Fig. 1-D). Under favorable conditions, the spots slowly enlarge, becoming irregular, water-soaked, and sunken with light tan centers and darker brown borders. The yellow, diffused margin enlarges around the spots; under humid conditions, the spots are characteristically soft and full of water. When the pathogen invades the large veins and midrib, advancement into the petiole and stem often follows, resulting in yellowing and total collapse of the leaf. On occasion large, water-filled blisters form on the under-leaf surface. Under less favorable conditions, the spots become dry and brittle and develop a torn or ragged appearance.

Spots on *D. picta* 'Rudolph Roehrs' are less clearly defined, being more water-soaked in appearance and soft, later becoming dry and papery. The advanced margin remains hydrotic and large areas of the leaf usually are involved.

*Plant inoculations.*—When dieffenbachia stems were

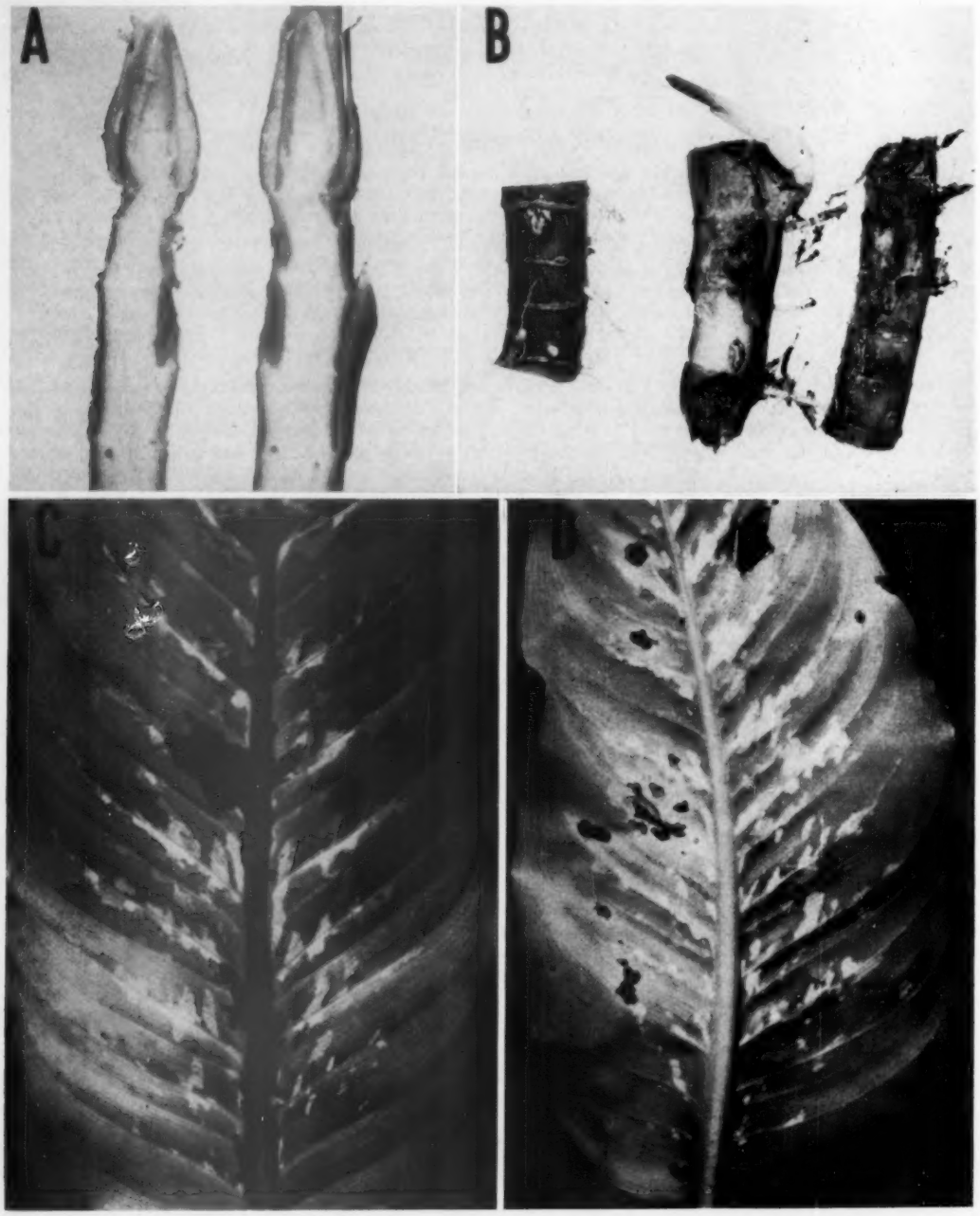


**Fig. 1.** Symptoms of bacterial stem and leaf rot of *Dieffenbachia.* **A)** Stem cut open to show early stage of infection. **B)** Natural infection on cane sections at right and healthy cane at left. **C)** Undersurface of *D. picta* var. *amoena* leaf showing soft rot lesions resulting from a systemic stem infection. **D)** Undersurface of *D. picta* var. *amoena* showing typical leaf spot lesions.

inoculated using a sharp pointed scalpel contaminated with the pathogen, typical soft rot lesions developed within 4-7 days, but many plants were not killed even after 1 or more months. When freshly cut dormant cane sections were inoculated by placing them in a dilute water suspension of the bacterium prior to planting in moist peat moss, a soft rot developed on the cut surfaces within 24-48 hours. After 7-10 days, a high percentage of the canes was rotted completely. Inoculated cane sections remained firm and without visible decay when allowed to remain 3 days on the laboratory bench but rotted rapidly when planted later in moist peat moss.

Inoculated petioles developed a localized necrosis around the point of inoculation within 48 hours, and collapsed after 7-10 days. When the areas between the large veins of mature leaves were injured and then inoculated, no infections resulted. When bacteria were introduced into very young leaves prior to unfolding, with the aid of a hypodermic syringe, small leaf spots developed. Thus, it was shown that leaf infections occurred only when the leaves were young and prior to unfolding, whereas mature leaves were not susceptible.

*Conditions favoring the disease.*—The conventional conditions under which dieffenbachia plants are grown, including heavy shade, high humidity, high temperature, poor ventilation, and crowded conditions, are ideal for the pathogen. Seasonably, disease conditions are optimum during the rainy summer months in south Florida. In winter, when temperatures are lower and rains less frequent, disease development is slowed down but not entirely halted.

When stems of dieffenbachia were artificially inoculated and placed in an infection chamber at high relative humidity for 1 week, disease progress was slow at 65°F, moderate at 75°F, and rapid at 85°F.

Frequent rains or syringing of the foliage favors leaf spot development. Under dry, less humid conditions, the spots become dry and brittle. Once the bacterium enters the vascular system, however, moisture seemed much less critical.

*Dissemination.*—Localized spread of the pathogen in the nursery is believed to occur on contaminated knives, tools, carrying trays, *etc.*, used in cutting and handling dormant canes, or when tip cuttings are taken for propagation. Cane sections may appear healthy at time of planting, yet be contaminated, as shown by development of the rot when such sections are planted in sterilized peat moss. Unrooted tip cuttings frequently become infected when planted in contaminated soil. Since a high percentage of plants having stem lesions also exhibited leaf infections, it is believed that bacteria are spread from infected cane to the new leaves by overhead watering and rains.

*Varietal susceptibility and host range.*—Only dieffenbachia has been found naturally infected with the soft rot bacterium in commercial foliage plant nurseries. Plants of *Dieffenbachia bausei* Hort. var. *Chiswick*, *D. picta*, *D. picta* 'Rudolph Roehrs', *D. picta* var. *amoena*, *D. picta* 'Roehrs Superb,' *D. memoria-corsii* Fenzi, and 2 local selections, referred to as 'Arvida' and 'Hoffmani' in the trade, were susceptible when artificially inoculated.

A representative collection of cultivated aroids and other plants commonly found in foliage plant nurseries were stem-inoculated and observed for a period of 3 weeks. The following were found nonsusceptible: *Monstera deliciosa* Liebm., *Philodendron oxicardium* Schott, *P. panduraeforme* Kunth, *P. selloum* Koch, *Alocasia* sp., *Peperomia obtusifolia* A. Dietr., *Schefflera actinophylla* Harms, *Syngonium podophyllum* Schott, *Scindapsus aureus* (Lind. & Andre) Engl., *Aphelandra squarrosa* var. *Leopoldii* Van Houtte, *Aglaonema robelinii* (Lind.) Gentil, *A. modestum* Schott, and *Spathiphyllum kochii* Engl. & Krause. Other plants found nonsusceptible were *Chrysanthemum morifolium* Ram., *Antirrhinum majus* Linn., and *Pelargonium* sp. A rot developed when leaf petioles of *Zantedeschia aethiopica* (L.) Spreng., and stems of *Aglaonema pictum* Kunth were inoculated and kept in a moist chamber for 48 hours.

*The pathogen.*—Six isolants of equal pathogenicity from stem and leaf lesions were selected for further study. The purity of each culture was assured by making frequent dilutions in sterile water and selecting individual colonies. These were maintained on Bacto-nutrient agar. The methods followed were essentially those suggested elsewhere (4, 5, 15). The carbohydrates were sterilized by filtration and added to a basal medium to make a final concentration of 1% unless stated otherwise, and the organic acids at 0.15% concentration. Cultures of *Erwinia carotovora* (E-32), *E. atroseptica* (van Hall) Jennison (E-29), *E. chrysanthemi* (EC-16), and *E. aroideae* (Townsend) Holland (EA-12), obtained from W. H. Burkholder, Cornell University, were run simultaneously with the dieffenbachia pathogen.

*Morphology.*—In Congo Red smear preparations, the cells appeared as straight rods with rounded ends, occurring singly, occasionally in pairs, or in short chains. Cells were motile as evidenced by a diffused growth in Bacto-motility medium. The pathogen was gram-negative, and exhibited peritrichous flagella when stained using Leifson's (9) method. In Congo Red stain preparations, cells from 24-hour streaks on Bacto-nutrient agar, averaged 2.75 x .75 $\mu$ (1.61-3.57 x .17-1.07 $\mu$).

*Cultural characteristics.*—Moderate growth occurred on Bacto-nutrient agar within 18 hours. The streaks, characterized by wavy margins, were gray to cream in color, filiform in shape, and butyrous in consistency. In cultures 1-week-old or more, the color of the streak changed to light tan in the centers while the edges remained gray to cream colored. Growth on freshly prepared potato-dextrose agar was luxuriant after 24 hours, filiform, butyrous, cream to pale yellow with wavy margins. Gas frequently formed in this medium. Bacto-nutrient broth became turbid within 12 hours and a pellicle formed after 24 hours.

After 1 month, most isolates failed to coagulate milk, and litmus was only partially reduced. Little, if any, peptonization occurred and the medium be-

came slightly acid. In Clara's medium, the pathogen produced a moderate turbidity and light pellicle. A whitish turbid growth occurred in Fermi's solution. Krumwiede's triple sugar agar supported good growth and the medium turned yellow, then red (older isolants often failed to turn the medium red). Growth on deoxychloate agar was pink at first, but became pale yellow in 2 days; growth on bismuth sulfide agar was scant (often lacking) and the colonies dark brown with a metallic sheen.

*Physiological reactions.*—The pathogen was aerobic when grown in deep cultures of nutrient-dextrose agar. Growth occurred in Bacto-nutrient broth supplemented with 3 and 5% NaCl, but not at 7%. A faint turbid growth occurred in nutrient broth within 1 week at 40° and 9°C.

Growth of the pathogen in a 12% gelatin-nutrient medium at 20°C was observable after 48 hours. Liquefaction was slow to moderate with less than 33% of the medium liquefied after 1 month. A solidified sodium polypectate medium, however, was liquefied within 24 hours. In Koser citrate medium, faint growth occurred at pH 3.4 and 8.4.

The dieffenbachia bacterium produced a strong positive test for indole when grown in 1% Bacto-tryptone broth, using Kovacs' reagent. Most isolates of the bacterium were methyl red positive after 1 and 3 days, changing to weakly positive or negative on longer incubation. Acetylmethylcarbinol was formed after 3 days as indicated by a positive Voges-Proskauer reaction. A strong test for nitrite resulted when a few drops each of sulfanilic acid and alphanaphthylamine reagents were added to Bacto-nitrate broth cultures. A negative test for $H_2S$ resulted when the pathogen was grown in 1% Bacto-tryptone broth. A strong test for $H_2S$ resulted, however, when the pathogen was grown in 1% BBL thiotone. Growth was fair in Ayers, Rupp, and Johnson medium containing ammonium salts and sucrose. The pathogen utilized asparagine as a combined carbon and nitrogen source.

When grown in a synthetic medium (Koser's medium minus the citrate plus bromthymol blue and 0.1% Bacto-peptone), the pathogen produced acid and gas from the following compounds: arabinose, rhamnose, xylose, glucose, fructose, galactose, mannose, sucrose, cellobiose, mannitol, ribose, 0.5% galacturonate, 0.5% aesculin, and salicin. Acid, but no gas, formed in media containing inositol, glycerol (weak), sorbitol (trace after 4 days, changing to neutral in 10 days), 0.5% gluconate, glucuronolactone, and ethanol (weak). No change occurred after 1 month in media containing lactose, maltose, trehalose, melibiose, mucate, adonitol, raffinose, melezitose, dextrin, dulcitol, inulin, and alpha-methyl glucoside. An alkaline reaction resulted when the pathogen was grown in a basal medium containing the sodium salts of citric, malonic, and tartaric acids; media containing hippuric or uric acids remained unchanged after 1 month.

A 0.2% soluble starch medium was not hydrolyzed after 2 or 7 days' incubation. Colonies of the pathogen grown on spirit blue agar containing cottonseed oil imparted a bluish color to the medium, indicating fat hydrolysis. The cultures were urease negative and catalase positive. All cultures formed gas in formate-ricineolate broth and lauryl-tryptose broth but not in Bacto-lactose broth.

When Bacto sensitivity discs containing known concentrations of antibiotics were placed on Bacto-heart-infusion agar seeded with the bacterium from dieffenbachia, growth was inhibited by 5, 10, and 30 μg each of Aureomycin (chlortetracycline), Chloromycetin (chloramphenicol), Terramycin (oxytetracycline), and Achromycin (tetracycline). Di-hydrostreptomycin and streptomycin each at 2, 10, and 100 μg, Penicillin at 2, 5, and 10 USP units, Polymyxin B (aerosporin) at 50, 100, and 300 μg, and Erythromycin at 2, 5, and 15 μg inhibited growth of the pathogen.

*Cross-inoculation tests.*—The dieffenbachia pathogen produced a typical soft rot of several fleshy vegetables including slices of potato tubers, radishes, cauliflower, onions, carrots, celery, turnips, and cabbage when inoculated and placed in a moist chamber at 30°C for 18 hours. In addition, inoculated calla rhizomes and caladium tubers were partially rotted after a 3-day incubation in a moist chamber.

In slathouse tests, stems of *D. picta* inoculated with either *E. chrysanthemi, E. carotovora* f. sp. *parthenii* Starr, *E. carotovora, E. aroideae,* or *E. atroseptica* failed to develop soft rot symptoms after 4 weeks. Stems inoculated with the dieffenbachia pathogen developed typical symptoms after 4 days.

*Taxonomic relationships.*—The morphological, biochemical, and cultural characteristics of the pathogen place it in the genus *Erwinia* (2). Its ability to produce a soft rot of dieffenbachia stems and leaves, and certain fleshy vegetables, and its action on polypectate medium suggest a close relationship to the soft rot group of organisms, or *Pectobacterium* group, according to the classification of Waldee (17). It was noted in early tests by McFadden (12), that the dieffenbachia pathogen was more closely related to *E. chrysanthemi* than to the other species studied. Munnecke (14) came to the same conclusion, and chose to call the organism a strain of *E. chrysanthemi.*

Several proposals have been advanced in recent years concerning the taxonomy and nomenclature of the *Erwinia* group. Dowson (4), and Hellmers (7) have adapted the genus *Pectobacterium* for those pathogens causing soft rots in plants. Dowson (4) reduced *E. chrysanthemi* in 1957 to a form species (*formal speciales*), *P. carotovorum* f. sp. *chrysanthemi.* Graham and Dowson (6) recently changed the chrysanthemum pathogen to *P. carotovorum* var. *chrysanthemi.* Burkholder (3) does not consider *Pectobacterium* a sound and useful genus and cites certain inconsistencies in following such a classification. The indiscriminate use of varietal and form species names is likewise discouraged, especially when used interchangeably. Hellmers (7), recognizing the similarity between *E. carotovora* f. sp. *parthenii* and *E. chrysanthemi,* chose to raise the former to *P. parthenii* and reduce the latter to *P. parthenii* var. *chrysanthemi.* As pointed out by Burkholder, however, *E. chrysanthemi* must remain the legitimate

TABLE 1. Differences between *Erwinia dieffenbachiae* and *E. chrysanthemi*

| Test or medium | *E. dieffenbachiae* | *E. chrysanthemi* |
|---|---|---|
| Lactose (1 month) | not utilized | utilized in 2 wks. |
| Purple lactose agar | unchanged | turns pale yellow in 2 wks. |
| Lactose broth | no gas | gas |
| Raffinose | not utilized | acid and gas |
| Melibiose | not utilized | acid and gas |
| Inulin | not utilized | acid and gas |
| Litmus milk | not coagulated (majority) | coagulated |
| Indole | strongly positive | negative or feeble |
| *Chrysanthemum morifolium* (var. Bluechip) | not pathogenic | pathogenic |
| *Dieffenbachia picta* | pathogenic | not pathogenic |

species, according to the International Code of Botanical Nomenclature (8). It should be stressed also that *E. chrysanthemi* varies in at least 8 important characters from *E. carotovora*, and therefore is in no sense a form species or variety of the latter.

The author is in general agreement with Burkholder, who maintains that nomenclature should be utilitarian, and that a specific epithet be used for a specific disease-producing bacterium. *E. chrysanthemi* and the dieffenbachia pathogen failed to cross-infect on their respective hosts, *Chrysanthemum morifolium* and *D. picta*. The most unique difference between the dieffenbachia bacterium and the remaining soft rot pathogens is its inability to utilize lactose. In this respect, the pathogen resembles certain members of the *Proteus* group. The dieffenbachia organism, however, does not decompose urea. In addition to its inability to utilize lactose, the dieffenbachia pathogen consistently varies from *E. chrysanthemi* in certain other important characters (Table 1). No doubt further differences would be found if other tests were performed. For these reasons the dieffenbachia bacterium should be considered a distinct species; the name *Erwinia dieffenbachiae* sp. n., therefore, is proposed.

*Control.*—Agri-mycin 100 (streptomycin 15.0% + oxytetracyline 0.5%) when used as a 30-minute preplanting liquid dip treatment gave satisfactory control without injury, but failed to completely eradicate the disease in inoculated single-eye cane pieces. Increasing the concentration of Agri-mycin 100 from 400-1,000 ppm active failed to give a corresponding increase in disease control. Control was unsatisfactory when inoculated cane sections were lightly dusted with either 5% active Agri-mycin 100, Agri-mycin 500 (streptomycin 1.75% + oxytetracycline 0.18% + tribasic copper sulfate 42.4%), or Agri-Strep (streptomycin sulfate 37%), prior to planting in sterilized moist peat moss. Dieffenbachia stem rot also was reduced but not completely controlled when inoculated cane pieces were immersed 30 minutes in hot water. *Dieffenbachia picta* 'Rudolph Roehrs' tolerated water temperatures up to and including 49°C, whereas *D. picta* var. *amoena* withstood 51°C without apparent injury. Higher temperatures were detrimental to dieffenbachia cane. Munnecke (14) obtained similar results and found disease control best using a combination of treatments; namely, a 15-minute cane soak in Agri-mycin 100 at 200 ppm, followed by immersion in hot water at 49°C for 40-60 minutes, depending on cane diameter. This general method also was found satisfactory in south Florida.

Extreme care is needed in handling the cane after treatment to avoid recontamination. Trays, tools, gloves, *etc.*, used in handling treated cane, should be disinfected at frequent intervals and the cane planted directly in a sterilized medium. The application of Agri-mycin 100 at 200 ppm active at 10-day intervals was found safe and may help reduce leaf infections in nurseries where this phase of the disease is destructive.

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# INFLUENCE OF ENVIRONMENTAL TEMPERATURES ON METABOLIC ALTERATIONS RELATED TO DISEASE RESISTANCE IN SWEET POTATO ROOTS INFECTED BY BLACK ROT

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Part 31 of a study of phytopathological chemistry of sweet potato with black rot.

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SUMMARY

Symptoms and induced metabolic alterations of sweet potato root tissues infected by *Ceratocystis fimbriata* were investigated at 4 temperatures, 20°, 25°, 30°, and 34°C. Host resistance was influenced markedly by environmental temperatures; more pronounced at 30° or 34°C than at 25° or 20°C. Optimal temperature for pathogen growth in culture was 25-30°C. The respiratory rate and the rate of ipomeamarone synthesis in infected tissue were increased in the range 20-30°C, but showed no difference at 30-34°C. Each ratio of respiratory activity and ipomeamarone synthesis of 30°/25°C was much higher than that of 25°/20°C. This feature also was observed in the case of polyphenol synthesis, though the ratio of the amount at 30°/25°C was almost the same as that at 25°/20°C. Polyphenol oxidase activity of infected root tissue at 30°C was significantly higher than that of uninfected tissue or infected tissue at 25°C. Thus, these metabolic alterations were more pronounced in the infected tissue incubated at 30° or 34°C than that at 25° or 20°C. The higher resistance at 30° or 34°C may result from metabolic changes of the host tissue in response to the fungal penetration. Influence of environmental temperatures was more conspicuous in the resistant variety than in the susceptible variety.

Introduction.—Biochemical and physiological study of plant disease has received increasing attention of many researchers. Alterations of metabolism in the host tissue in response to the pathogenic infection have been recognized, in many cases, as rather common features of both facultative and obligate parasitism (7, 9, 10, 16, 25).

We have long been working on a study of sweet potatoes infected by the black rot fungus (*Ceratocystis fimbriata* Ell. & Halsted) to reveal the mechanism of metabolic alterations in the infected root tissue (33, 34). Notable increase in respiratory rate and synthesis of polyphenol and coumarin substances has been observed in the sound tissue adjacent to the injured region (2, 28, 29). Formation of ipomeamarone in the injured tissue was found by Hiura (13), and constitutes an unique picture of this particular disease. Ipomeamarone is a postinfectional metabolite and exhibits a potent antipathogenic action toward the fungus (3, 13, 32). Recently an analytical study on its formation was carried out (6). Other major metabolic changes are 1) increase in organic phosphate and concomitant decrease in inorganic phosphate (2), 2) activation of enzymes and enzyme systems (30), and 3) change in nitrogen metabolism including synthesis of immunochemically specific protein constituents in infected tissue (4, 36).

All those metabolic changes are considered reactions of the host tissue induced by infection, and are believed to constitute some part of the host resistance. Our observation on black rot disease indicates that the magnitude of increased respiratory rate and the rate of ipomeamarone synthesis are roughly parallel to the degree of resistance action of the host plant, and that the polyphenols take part in the formation of chemical barricade, although their quantity is not always proportional to the degree of resistance (33, 34). Experimental evidence has been accumulated also in other plant diseases to verify the dominant role of the dynamic host metabolism in the defense mechanism of infected plants (8, 22, 24, 26, 27, 34).

Some workers already have indicated that plant resistance to pathogen penetration is influenced by environmental temperatures (15, 23). This is indeed the case in black rot disease of sweet potatoes. Lauritzen (20) and Hemmi, *et al.* (12) found that resistance of

root tissue showed some distinct features at varied temperatures, higher at 30°C, compared with 23-27°C. None of this research, however, has dealt with the interrelationship between these phenomena and host metabolism.

The present paper deals with some experiments on the effect of environmental temperatures on metabolic alterations in sweet potato roots infected by *C. fimbriata*, aimed at explaining the change in resistance action of the host as influenced by temperature.

MATERIALS AND METHODS.—Sweet potato roots used in this study were cultivated at the farm of the Konosu National Agricultural Experiment Station, under the standard ground condition. After harvest, they were sent to us without any injury, and stored at 10°C until used. Two varieties, Norin No. 1 and No. 2, were used, respectively, as resistant and susceptible varieties. Standard procedure of spore inoculation of *C. fimbriata* on the surface of the host tissue and the incubation were previously reported (4, 6). Incubation temperatures were 20°, 25°, 30°, and 34°C, and relative humidity was kept at 98-100%. Caution was taken to avoid the evaporation of samples incubated at 34°C. Forty-eight hours after inoculation, the sound tissue (2-3 mm thick), adjacent to the injured region, was taken for manometric experiments (respiratory oxygen uptake and polyphenol oxidase activity) and polyphenol analysis. Injured tissue was subjected to ipomeamarone analysis.

*Measurement of growth of C. fimbriata.*—The following method was employed for the quantitative measure of the fungus growth: to each 10 ml of cultural medium composed of potato extract (boiled extract of 250-g potato tuber tissues with 1 liter of water) containing 2% sucrose was added 1 ml of spore suspension of *C. fimbriata* then incubated as a stand culture at each of the regular temperatures: 20°, 25°, 30°, and 34°C. The fungus spores as inoculum were collected by centrifugation from the shaking culture. After 6 days of incubation, the mycelium was collected and the dried material weighed, after thorough washing and desiccation. In each case, 4 flasks were incubated in parallel; average mg-dry weight of mycelium/flask was represented as a magnitude of the fungus growth.

*Measurement of respiratory rate.*—Thin slices (0.5 mm thick, 7 mm in diameter) were prepared from inner tissue adjacent to the injured region and also from healthy roots without any treatment. Warburg manometric methods were used to measure respiration. Each flask contained 20 slices of tissue in 2 ml of 0.03 *M* phosphate buffer (pH 5.5) containing 2% sucrose. Measurement was carried out at the same temperatures as those of incubation of root tissues.

*Measurement of polyphenol oxidase activity.*—Simple homogenization of sweet potato root tissue causes an oxidation of polyphenolic substances; the resulting quinones inhibit several enzyme activities. Measurement of polyphenol oxidase activity in the infected tissue was markedly hindered by such an event (4), and the following procedure was established to overcome the difficulty: 100 ml of pre-chilled pure acetone (—30—40°C) containing 0.2% ascorbic acid were added to 5 g of the sound tissue adjacent to the injured region and were homogenized promptly with a Waring Blendor. After rapid filtration, the acetone powder was repeatedly washed with 100 ml each of cold pure acetone and ether; then dried over phosphorus pentoxide *in vacuo*. To the acetone powder were added 15 ml of cold distilled water; extraction was performed in a cold mortar. After adjusting to pH 7.0, it was centrifuged at 500 *g* for 10 minutes. The clear yellow supernatant was used as an assay of polyphenol oxidase. One ml of 0.1 *M* phosphate buffer (pH 7.0), 1.0 ml of 0.01 *M* methyl caffeate, and 1.0 ml of 0.04 *M* ascorbic acid were added to main cell of the flask; 0.1 ml of the above enzyme preparation was placed in the side arm. After 10 minutes of equilibration, the enzyme preparation was tipped from the arm and reading was begun. All measurements were carried out at 30°C.

*Chemical analyses of ipomeamarone and polyphenol substances.*—Details of the analyses of impomeamarone and polyphenol substances were given in the preceding papers (5, 6). For ipomeamarone analysis, an ethanol extract of the injured tissue was applied to the silica gel chromatostrip and its synthetic pattern was examined both qualitatively and quantitatively. For polyphenol analysis, an ethanol extract of the sound tissue adjacent to the injured region was applied to the alumina column, as devised by Zucker and Ahrens (37).

RESULTS.—*Growth of C. fimbriata.*—Growth rate of the fungus examined under 4 different temperatures (Fig. 1) indicates that the mycelial formation in the cultural medium was increased by raising the temperature from 20° to 30°C, but was drastically suppressed at 34°C. Thus, there seems to be no great difference between the fungus growth at 25° and that at 30°C. The optimum temperature for growth is 25-30°C. These observations are in agreement with the previous reports by Lauritzen (20) and Hemmi, *et al.* (12).

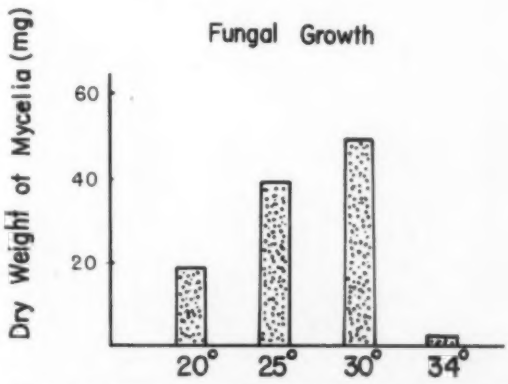


**Fig. 1.** Amount of mycelium produced by cultures of *Ceratocystis fimbriata* in 6 days of incubation at different temperatures.

*Infection symptoms.*—In order to evaluate the magnitude of resistance action of the host tissue, infection symptoms were investigated under varied temperatures for the following items: magnitude of the browning reaction in the injured tissue, cork layer formation in the boundary region between sound and injured tissues, and formation of aerial hyphae and perithecia of the fungus on the tissue surface. The following are considered to be typical of resistance-action: 1) intense browning, 2) thinness of the injured region, 3) marked disturbance of the formation of aerial hyphae and perithecia, and 4) rapid formation of cork layer. When Norin No. 1, the resistant variety, was infected at 34°C, the injured region showed a strong browning, but it was restricted to the surface region, about 0.1 mm thick. Formation of a cork layer occurred very rapidly, and neither aerial hyphae nor perithecia were formed. At 30°C, the injured region showed browning, but its thickness was about 0.1-0.2 mm. Cork layer formation was not pronounced, and aerial hyphae were not formed. Perithecia also were not formed. On the other hand, when infected at 25°C, necrotic browning was less marked than that at 30°C, and thickness of the injured region was about 0.5 mm. The cork layer was not formed, but both aerial hyphae and perithecia were formed. At 20°C, there was a little less browning than at 25°C, thickness of the injured region was about 1 mm, and other features were similar to those at 25°C. From these results, it can be concluded that the root tissue of Norin No. 1 exhibits stronger resistance action when infected at 34° and 30°C than at 25° and 20°C. We may exemplify the relation of the host resistance to temperature as follows: 34°> 30°>> 25°> 20°C. Infection symptoms of Norin No. 2, the susceptible variety, were significantly different from those of Norin No. 1. At the initial stage of infection, the fungus

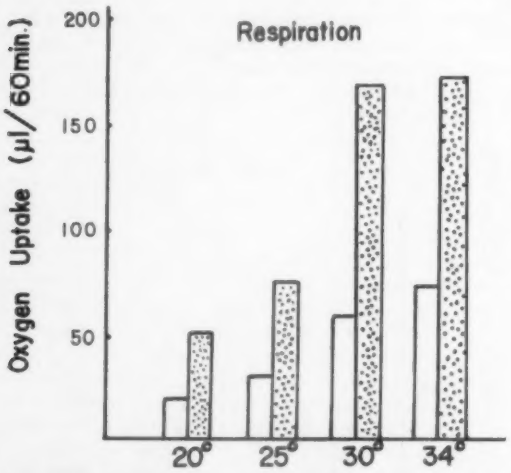


**Fig. 2.** Respiration of sweet potato tissue from uninfected roots (unspotted bar) and of sound tissue from region adjacent to injured area of roots infected with *Ceratocystis fimbriata* (spotted bar). Samples were taken 2 days after inoculation.

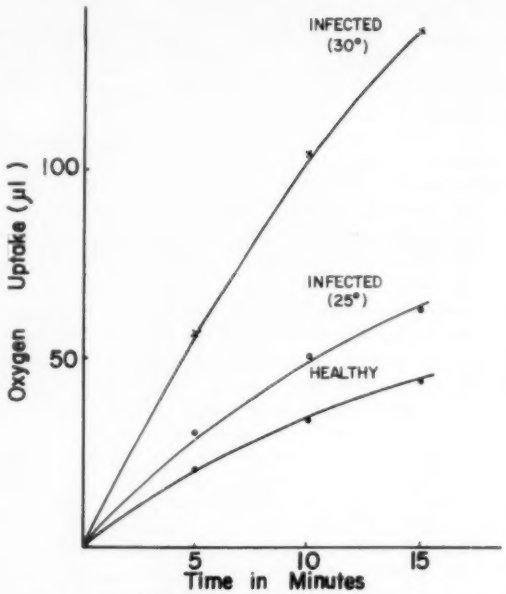


**Fig. 3.** Polyphenol oxidase activity in acetone powders prepared from equivalent amounts of tissue from diseased and healthy sweet potato roots. Diseased roots were inoculated with *Ceratocystis fimbriata* and incubated for 2 days before powders were prepared. Incubation temperature for the healthy roots was 30°C.

growth in the tissue was less marked, but the eventual penetration was more severe. Consequently, thickness of the injured region was greater than that in the resistant variety. Furthermore, the browning reaction did not occur on a large scale at 4 different temperatures; this feature is likely to be connected with a weak resistance of Norin No. 2.

*Respiratory increase in infected tissue as a function of incubation temperature.*—Respiratory rates of adjacent sound tissues were measured at the same temperatures as those used in incubation. Measurements were carried out also for the healthy root tissue without any treatment in order to compare the temperature effect on the basal respiration with that on the increased respiration induced by the fungus infection. Results (Fig. 2) clearly indicate that respiratory rates of the infected tissues were more than doubled at all 4 temperatures in comparison with those of untreated healthy tissue. The rates were highest at 30° and 34°C for both diseased and healthy tissues. The ratio of repiratory activity at 30°/25°C was much higher than the ratio of the activity at 25°/20°C. This relationship was more marked in infected than in healthy tissue.

*Polyphenol oxidase activity of infected tissue.*—When infected at 30°C for 2 days, polyphenol oxidase activity of infected root tissue was significantly higher than that of the uninfected healthy root tissue (Fig. 3). Enzyme activity of the infected tissue at 25°C was more or less the same as that of the uninfected healthy tissue.

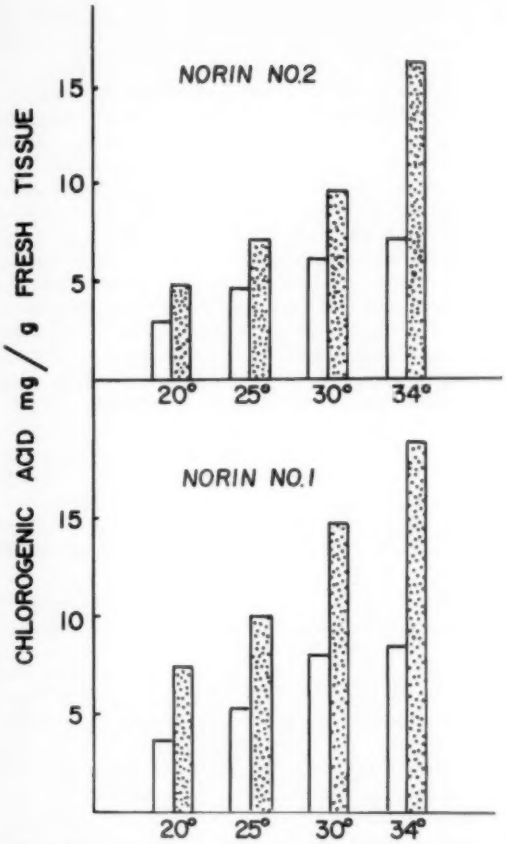


**Fig. 4** Chlorogenic acid levels in tissue from *Ceratocystis fimbriata* infected sweet potato roots. Roots were inoculated and then incubated at the indicated temperatures for 48 hours (unspotted bars) or for 72 hours (spotted bars) before determinations were made.

*Alteration of polyphenol substances in infected tissue.*—Marked increase of polyphenol substances, including chlorogenic acid, isochlorogenic acid, and other similar compounds, has already been observed in the infected tissue (28). Therefore, in this report, comparison has been made of resistant and susceptible varieties under varied incubating temperatures. The magnitude of these substances synthesized in the infection period of 48 hours was slightly higher in the resistant variety than in the susceptible one, but the rate of synthesis in the next 24-hour period (48-72 hours) was more marked in the former variety (Fig. 4). It should be noted that the level of polyphenols in the uninfected healthy roots is very low (less than 0.5 mg/g) at 4 temperatures, and a steady increase occurred in infected roots of both varieties when environmental temperature was raised. Thus, polyphenol formation is characteristic of the postinfectional metabolism, regardless of the incubation temperature. Increase of polyphenol substances in the resistant variety paralleled development of necrotic browning in the host tissue; browning was meager, however, in the susceptible variety.

*Synthetic pattern of ipomeamarone and other related substances in infected tissue.*—Recent analytical experiments have demonstrated that the synthesis of ipomeamarone and other analogous Ehrlich reagent-positive terpenoids reached the maximal level at about 72 hours after infection (1, 6). Chromatographic methods were applied to determine the synthetic pattern of these postinfectional metabolites as a function of temperature at 48 hours after infection (Fig. 5). Rate of synthesis was much greater in the resistant variety than in the susceptible one under all 4 temperatures, a different picture from that of polyphenol synthesis. In the former variety, their formation at 34° exceeded that at 30°C; the reverse was true of the latter. Under the lower temperatures, synthesis was low; at 20°C, a minute amount was detectable in both varieties, indicating slow biosynthesis. Ipomeamarone, a major component of those furanoterpenoids, was analyzed quantitatively to reveal more precisely the pathogenically induced metabolic change (Fig. 6). In the resistant variety, the synthesis in the initial 48 hours showed little difference at 30-34°C, but their levels

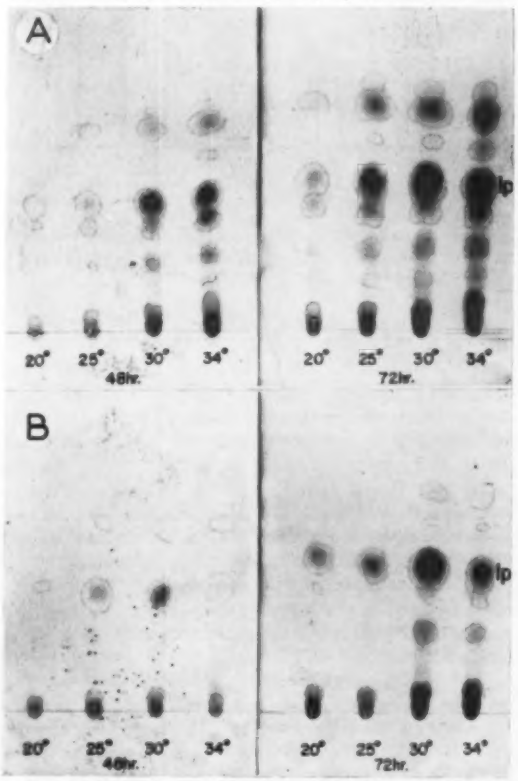


**Fig. 5.** Synthesis of ipomeamarone and other Ehrlich reagent-positive terpenoids examined by chromatostrip technique. **A)** Resistant variety Norin No. 1. **B)** Susceptible variety Norin No. 2.

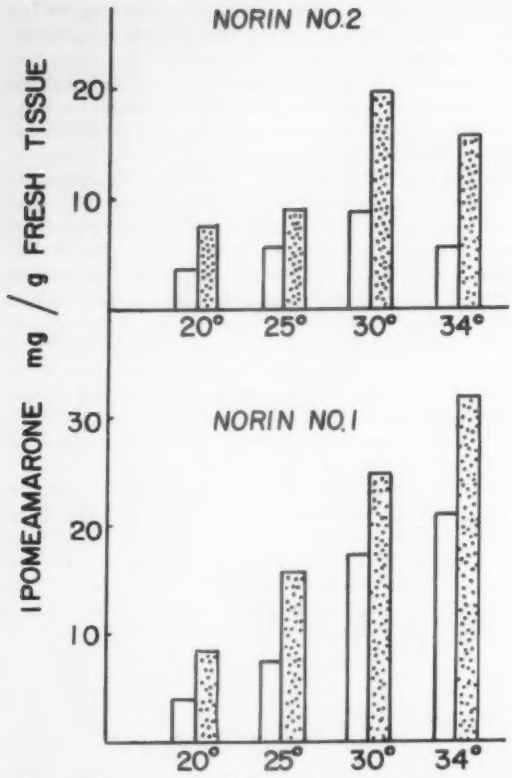


**Fig. 6.** Ipomeamarone levels in tissue of sweet potato roots infected with *Ceratocystis fimbriata.* Roots were inoculated and then incubated at the indicated temperatures for either 48 hours (unspotted bars) or 72 hours (spotted bars before determinations were made.

were considerably higher than those at 20° and 25°C; the ratio of the amount at 30°/25°C was much higher than the ratio at 25°/20°C. In a subsequent 24-hr period (48-72 hours), further increase occurred at all 4 temperatures, but the rate of synthesis decreased quite considerably in the tissues infected both at 30° and 34°C, compared with those at 20° and 25°C. In the susceptible variety, 30°C was the favorable temperature for ipomeamarone formation and the less marked synthesis was observed at 34°C. In the initial 48 hours, the synthetic magnitude was largest at 30°C, but not much different from that at other temperatures. Rate of synthesis in a subsequent 24 hours, however, increased further in the tissue infected at 30° and 34°C, but not at 20° and 25°C.

Discussion.—Our present experiments show that the resistance of sweet potato roots to *C. fimbriata* is more pronounced when infected at 30° than at 25°C. On the contrary, the growth rate of the pathogen on a cultural medium was slightly higher at 30° than at 25°C. Thus, it can be said that there is not much difference in the potentiality of penetration of the black rot fungus into the host cells at the 2 different temperatures, 25° and 30°C. Apparent difference in the resistance action of the infected root tissue can be seen in the effect of temperature on the metabolic action of host tissue in response to fungus infection. In the several varieties of sweet potato roots, some resistant and others susceptible to *C. fimbriata,* magnitude of the augmented respiratory rate and of ipomeamarone synthesis in the infected tissue was roughly parallel to the degree of resistance. The close connection of these patterns and the defense mechanism in host tissue were inferred (33, 34). The present information concerning the metabolism of the resistant variety showed that the respiratory rate and ipomeamarone synthesis were more profound in the infected root tissue incubated for 48 hours at 30° than at 25°C. Higher resistance at 30°C might be interpreted by such remarkable metabolic change of the host plant in response to the fungus infection. In the susceptible variety, neither resistance action nor ipomeamarone synthesis in the initial 48-hour period showed much difference between 30° and 25°C. Yet, less resistance action of the host tissue at both temperatures coincides also with less pronounced ipomeamarone synthesis in the injured tissue.

Some workers have held that the polyphenol-polyphenol oxidase system plays an important role in resistance action of infected plants (11, 17, 18, 19, 24). Our investigation on the black rot disease also has indicated that the role of polyphenol-polyphenol oxidase system in the resistance action may perhaps lie in the formation of chemical barricade; formation of brown pigments is based on the oxidation of polyphenols by polyphenol oxidase (28). In this connection, it should be emphasized that the polyphenol substances and polyphenol oxidase were more markedly increased in the infected tissue at 30° than at 25°C, but the ratio of the amount of polyphenols at 30°/25°C was not remarkable as compared with the amount of ipomeamarone production.

Studies on the ipomeamarone synthesis may reveal the biochemical basis of resistance action. Its pattern of synthesis as influenced by the environmental temperature may give particularly valuable information. Indeed, the more marked ipomeamarone synthesis and pronounced resistance features in infected root tissue incubated at 30-34°C are considered evidence for the actual participation of ipomeamarone in resistance action. A previous examination showed that injury of the host cells by fungal penetration was the essential step in initiating ipomeamarone synthesis in host tissues (35). The biochemical reaction causing cell injury might be a rate-limiting step for ipomeamarone formation and other metabolic changes, and be greatly stimulated by raising the environmental temperature. From the pathological viewpoint, injury, ipomeamarone formation, and browning constitute the processes of hypersensitive death. All are commonly observable in the resistant varieties. Ipomeamarone showed an inhibition of the host metabolism itself (28), and was assumed a factor in stimulating the browning of the

injured host cells (33, 34). Suzuki, *et al.* emphasized the role of ipomeamarone as a cell-killing factor (26). Also, ipomeamarone exhibits a powerful antipathogenic action against *C. fimbriata* and is considered an entity of the fungistatic principles produced by the infected root tissue (3, 26). Elucidation of the post-infectional, low-molecular compound having such an activity has not been well established. Müller's phytoalexin has given the first convincing evidence along the line though its chemical nature remains unknown (21, 22, 23). Synthetic pattern and local distribution of ipomeamarone in the infected sweet potato roots are similar to the description of Müller's phytoalexin (21, 22, 23). Jerome and Müller (14) have shown that pretreatment of pea seeds at 30°C resulted in a decreased output of phytoalexin after exposure of the tissue to the infection by *Sclerotinia fructicola* and discussed the "biological" nature of phytoalexin.

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# SOME EFFECTS OF TEMPERATURE DURING THE COLONIZATION PERIOD OF BEAN RUST

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SUMMARY

Controlled environment rooms were used to determine the effects of temperature on symptom development and sporulation during the colonization period following penetration of Pinto 111 beans by *Uromyces phaseoli* var. *typica* (Reb.) Wint. In the first experiments, 9 day-night temperature regimes between 90° and 60°F were used and results showed that the optimal range is 70-80°F. Higher or lower temperatures retarded first expression of disease and rate of symptom development. A temperature regime of 90° days and 80°F nights prevented symptom expression but the parasite remained alive for 10 days and could resume activity at lower temperatures.

A local necrosis developed when infections of certain ages were exposed to continuous 90°F for 5-7 days. Infections 96 and 120 hours old developed the local necrosis, those 72 hours old were killed, and those over 120 hours old became static without evidence of local necrosis.

Eight hours at 90°F following penetration retarded full development of symptoms by at least 1 day. Generally, symptom development was retarded in proportion to the amounts of time 90°F was endured. Night temperatures below 70°F had a similar effect. These reactions were responses to the actual temperatures encountered.

Effects of temperature on disease and pathogen development were studied during the period following penetration (postmoisture period). In the past, a great deal of attention has been paid to temperature effects, but seldom has temperature been isolated completely as the most important variable (3). The review which follows deals with only a small portion of this literature but serves to emphasize some of the problems involved.

REVIEW OF LITERATURE.—Optimum temperature range for rust development (colonization) and lengthening of incubation period with cooler temperatures is documented by Fromme (4) for *Puccinia coronata* (Pers.) Cda.; Peltier (10) for *P. graminis tritici* Eriks. & Henn.; Mains (6), and Syamananda and Dickson (17) for *P. sorghi* Schw., among others. Sempio (14) showed the same results for bean rust, although less clearly, because his controls received suboptimal temperatures. His scale of disease development types did not permit objective analysis; rate of disease progression was obscured by the method of recording data.

Sempio's report and those of Mohamed (7, 8) and Gordon (5), however, are among the few in the literature of rust diseases which describe infected plants subjected to treatments involving several temperatures between inoculation and sporulation. Using bean rust, Sempio noted that disease development was good up to 84°F, but the postpenetration temperatures of 90-93° for 4 days or 93-97° for 2 days "sterilized" infection. A bad wilt reaction occurred when plants were moved from 57-64° to 79-84°F 6 days after infection, but not when moved earlier. He stated that the more advanced the infection, the more sensitive the plant was to unfavorable environment, in this case over 84°F. No necrosis was mentioned in his paper.

Mohamed (7, 8) demonstrated that postinoculation temperatures quantitatively affect rust reaction. The longer inoculated seedlings of certain wheats were kept at high temperatures (85°F), the larger the uredia. Peturson (11) found some oats were susceptible to *Puccinia coronata* at higher temperature (77° F), resistant at more moderate temperature (70°), and immune at 57°. The reverse was reported by Newton, Johnson, and Brown (9), who found some wheats that were resistant to stripe rust at 78°F but susceptible at 54°F.

Mains (6) reported that no uredinia of *Puccinia sorghi* formed when postinoculation temperature was 86°F and that sparse mycelium was formed. He mentioned no necrosis. Syamananda and Dickson (17) and Dickson, Syamananda, and Flangas (2) observed a necrotic fleck induced by low temperatures in this

same host-parasite combination. Increased temperature brought about greater susceptibility.

Wei (18) reported for *Uromyces phaseoli* var. *typica*, with either highly susceptible or highly resistant varieties, that host reaction was little influenced by changes in postmoisture temperature. Temperature affected the relative proportion of each type of pustule produced in a mesothetic host. A typical mesothetic reaction occurred at 68°F which was near the host's optimum. At lower temperatures, the reaction between host and parasite was less "violent" as evidenced by a narrower necrotic zone; whereas at higher temperatures, the necrotic zone was larger. As temperature approached and reached 82.5°F, the relative number of susceptible reaction sori increased. Wei attributed this to a more rapid "aging" of the host leaves. The response of the variety used to the race used in the present studies was fully susceptible (Wei's type 3); according to Wei's study, there should have been no temperature effect on the protoplasmic reaction.

MATERIALS AND METHODS.—In the study of disease epidemiology, plants must be standardized, not only genetically, but environmentally. This can be done in controlled environment facilities (19). Plants should be inoculated with a fairly accurately known dosage /unit area of leaf (1, 12, 15). Moist chamber temperature should be controllable, although this was not important in the current phase of this project. Following penetration, several environments of small temperature range are necessary and facilities should allow rapid movement of plants among environments. Maintenance and reproduction of these environments are necessary for duplicating results. Of great importance is the ability to vary 1 factor while other important environmental factors are maintained.

Studies reported here were facilitated by the use of controlled-environment rooms. In each of 3 rooms, automatic controls provided a step-diurnal (12 hours-12 hours) temperature, humidity, and light regime. The dry-bulb temperature of 1 chamber was 70° days and 60°F nights; of a 2nd, 80° days, 70°F nights; and of the 3rd, 90° days and 80°F nights. During later studies, the 3rd chamber maintained a constant 90°. Saturation deficits for atmospheric moisture were equal in each room; days, approximately 5.54 mm, nights, approximately 3.15 mm. Light (750-900 ft-c) was supplied by "cool white" fluorescent lamps augmented by red tubes with a strong emission peak at 660 mμ.

Certified seed of Pinto 111 bean was obtained from the Bean Grower's Cooperative, Twin Falls, Idaho, and the same seed lot used throughout the studies. Seeds were planted 0.25 in. deep in sterilized greenhouse soil in 3-in. plastic pots in bottom-watering trays. The soil was Hagerstown fine sandy loam, 3 parts; sand, 1 part; and peat moss, 1 part. After planting and wetting the soil from the bottom, trays were placed in the 90°-80°F (day-night) chamber for 3-4 days, or until the cotyledonary crook was showing. They were then transferred to the 70°-60°F chamber; a red light rapidly straightened the crook. At these temperatures, a short, sturdy, nonclimbing plant developed. The 1st (sometimes the 2nd) trifoliate leaf was inoculated on 2-5-week-old plants; primary leaves were not used because their reactions were not typical of mature leaves.

A single-pustule isolate of the parasite from a local bean field was used without further standardization. Inoculum was maintained on living plants of Pinto 111 in the growth chambers, the greenhouse, and a Wardian case. To insure high germination levels, masses of dry spores were hydrated in a saturated atmosphere at room temperature for 48 hours (16).

Inoculating was done by using a settling tower and $CO_2$ pistol (1). Only 2% of spores could be reclaimed without electrification (675 v, d-c.) of the tower wall and gun muzzle. In addition, a wire was hung from top center just to the bottom of the tower (6 in. above the leaves) to give a torus of usable distribution in the circle on the board where leaves were exposed.

Sporefall was assayed by placing a water agar-coated slide next to each leaf inoculated or by placing 0.5 x 1 cm squares of agar on a bar lying on the leaf. These slides were later incubated with inoculated plants but in separate moist chambers so that germination could be assayed at the temperature at which germination and penetration of the leaves were occurring. Inoculum density in these studies was 400-1,000 spores/cm$^2$ and germination was 69-92%.

After inoculation, plants were placed in a moisture chamber with an air-water spray which did not blow directly on the plants. The spray in the chamber was started about 1 hour before plants were introduced and (to minimize runoff) was reduced after leaves were wet. Plants were left in the chamber 20-24 hours. About 3 ft-c (plant height) of light from an incandescent source were admitted through the glass chamber top to retard the characteristic drooping of bean leaves which occurs in the dark.

Sporulation was assayed by removing leaflets from 3 plants each day after sporulation had begun. Spores were washed and scraped with a rubber policeman from both leaflet surfaces in a known volume of water containing detergent. Spores in this suspension were counted with a haemocytometer and this figure was multiplied by the dilution factor. Subsequently, the sori on each leaflet were counted and this figure was used to calculate the yield of spores/sorus. The data for the 3 leaflets of each day were averaged.

All plants in all experiments were handled identically through the moisture period following inoculation. There was some variation from experiment to experiment in the time and temperature of the moisture period but the nature of the experiments did not make this an important variable.

EXPERIMENTS AND RESULTS.—Experiment 1 was a preliminary study to determine the extent to which temperature affected the rate of symptom development and sporulation. Day-night temperatures were respectively, 90-80°, 80-70°, 70-60°F. There were 9 plants/treatment for the rate of symptom development study

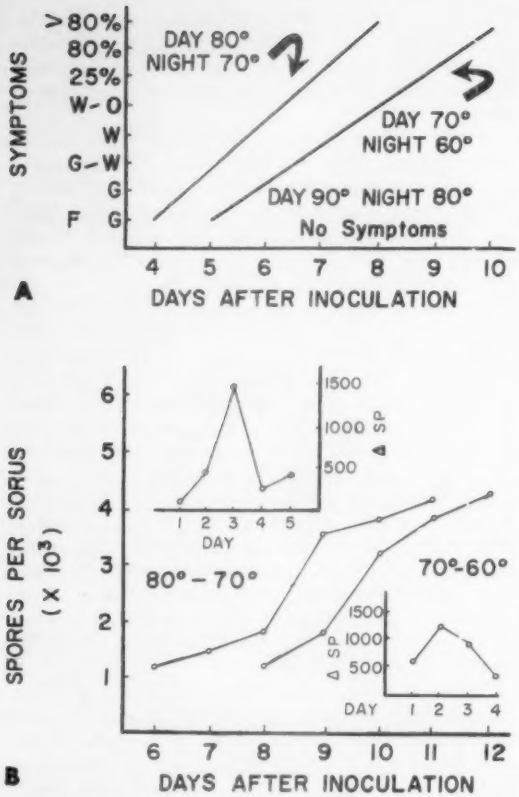


**Fig. 1.** Results of experiment 1. **A)** Rates of symptom expression development at day-night temperatures of 80-70° and 70-60°F. No symptoms were expressed at 90-80°F. Ordinate, from bottom to top, lists symptoms and signs recognized as explained in the text. **B)** Rate of development of sporulation expressed as numbers of spores/sorus at temperatures of 80-70° and 70-60°F. Inserts show daily increment of spores/sorus at each temperature after sporulation was started.

and 5 plants/treatment for the rate of sporulation study. Data were taken each morning for 12 days following inoculation.

In this experiment, as in all others, 8 stages of symptom development were recognized. The 1st consisted of a few, very faint, light green punctae on the lower surface (FG). These probably represent the 1st successful penetrations. About 24 hours later, punctae had increased about 10-fold and were much more distinct (many green, MG). After a lapse of 24-48 hours, depending on temperature, the punctae became green-white (G-W) and finally, white (W). Soon after, the orange of developing spores could be seen through the lower epidermis (W-O) and punctae were visible on the upper surface. Within the next 48 hours, again depending on temperature, the lower epidermis ruptured and stages of about 25 and 80% of pustules ruptured were recognizable about 24 hours apart.

Fig. 1-A shows that the coolest environment (70-60°F) retarded the appearance of symptoms by 1 day, and the attainment of full sporulation by 2 days. Symptom development was completely inhibited by the 90-80°F environment.

Fig. 1-B shows that development of sporulation was affected by temperature in about the same way as development of symptoms. The data must be considered only as indicative and not as absolute; other factors, such as leaf age, could affect number of spores produced. The inserted graphs show the unimodal curves obtained when the daily increment of spore (△ Sp) was plotted; these suggest a spore maturation period following 1st sporulation by 2-3 days, depending on temperature. These data indicate the probable epidemiological significance of only slightly adverse temperatures.

Experiment 2 included every temperature regime obtainable in the 3 rooms by moving plants on a 12-hour basis from 1 room to another. Besides the 3 cycles used in experiment 1, there were 2 constant-temperature treatments (70° and 80°F), 2 intermediate environments (90-70° and 80-60°F), and 1 extreme (90-60°F). There also was a diurnal reversal in which day temperature was lower than night temperature (70-80°F). This experiment was designed to determine if the disease response was a function of the actual temperatures or of their means. Data were taken daily following the beginning of symptom expression. There were 3 plants for each treatment.

The rates of development at day-night temperatures of 80-80°, 70-80°, and 70-70°F (Fig. 2-A) were almost identical and the degree of variation of data of several 80°-70°F experiments indicated that that environment was about equally efficacious. There was, however, some lag in disease development on plants in the 80-60°F environment, although not as great as at 70-60°F.

Again, plants in the 90-80°F room had not expressed symptoms by the 10th day after inoculation. The combination of high day temperature and cool night temperature (90-60°F) greatly retarded disease development; 7 days were required before stipling could be seen in transmitted light and 8 days before symptoms could be observed in reflected light. Warmer nights

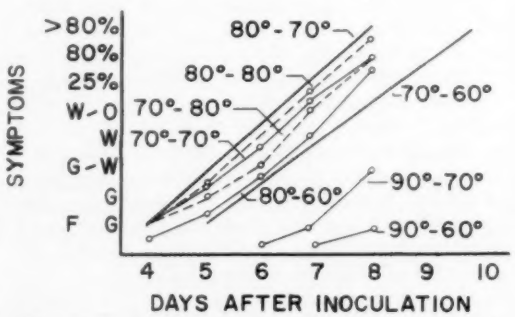


**Fig. 2.** Rates of symptom expression development observed on plants subjected to 9 temperature regimes. (Experiment 2.)

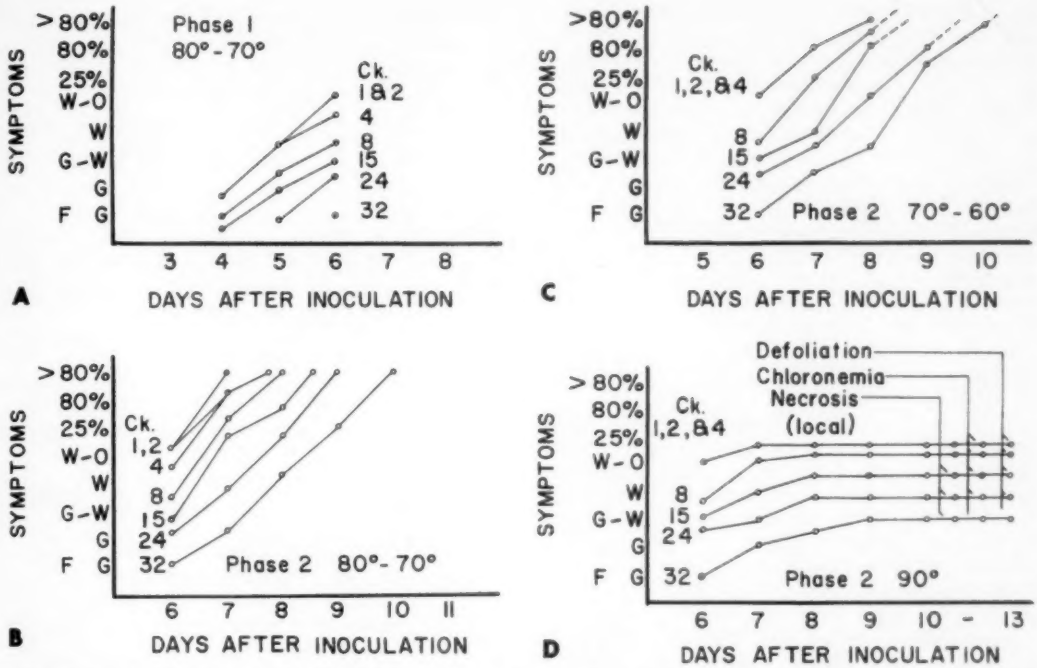


**Fig. 3.** Results of experiment 3. **A)** Results of phase 1, showing the retarding effect of various periods of heat. **B)** Progression of symptoms at 80-70°F during phase 2. **C)** Progression of symptoms at 70-60°F during phase 2. Note that in **C)** and **D)**, rates of development on all treated plants are approximately equal at each temperature indicating that the 90°F temperature encountered earlier had no residual effects. **D)** Results observed on plants at 90°F during phase 2. Defoliation and chloronemia were observed on all plants, necrosis on the 15-, 24-, and 32-hour plants only.

(90-70°F) allowed only slightly more rapid development.

The plants from the 90-80°F treatment, which showed no symptoms after 10 days in that environment, were transferred to the 80-70°F rooms to determine if the fungus was alive. Normal progression of symptoms was observed, followed by sporulation. There was, however, some reduction in the number of lesions. Repetitions gave similar results.

Because of certain high temperature responses noted in experiments 1 and 2, experiment 3 studied the sensitivity of the host-parasite interaction to high temperature. Immediately after completion of the moisture period, groups of 3 plants were exposed to 90°F constantly for periods of 1, 2, 4, 8, 15, 24, and 32 hours, after which they were placed in the 80-70°F room. Data were taken daily as to stage and rate of development of symptoms (Fig. 3-A).

Exposures of up to 4 hours, immediately following the moisture period, had little or no effect on the development of the host-parasite interaction. Exposures of 8, 15, 24, and 32 hours resulted in a progressive retardation of 1st symptom expression. When a conducive environment was restored, however, development of symptoms progressed at about the same rate as in the control experiment, as shown by the slopes of the lines.

By the 6th day after inoculation, plants in the various groups showed at least 5 different states of symptom development. At this point, phase 2 was initiated. One plant was left in the 80-70°F room, 1 was placed in the 90° chamber, and 1 in the 70-60°F chamber. Fig. 3-B illustrates what happened on plants that remained at 80-70°F. The data are simply a continuation of those plotted in Fig. 3-A. Symptom development continued on plants of all treatments. Net effect of the early exposures to 90°F was quiescent infections for periods of time roughly corresponding to the time of treatment. All infections progressed at about the same rate.

Plants in the 70-60°F chamber during phase 2 (Fig. 3-C) behaved similarly except that the rate of development of symptoms was slower as had been seen in experiments 1 and 2.

The responses of those plants placed in the 90° chamber during phase 2 were strikingly different (Fig. 3-D). Some further development of symptoms occurred for 48 hours after this treatment was begun and then stopped. Plants pretreated at 90°F for 15, 24, and 32 hours showed a distinct local necrosis of and surrounding the punctae. The necrosis was more advanced on the 32-hour plant which was least expressive of symptoms ("1st green" stage) when reintroduced to 90°F than on the 15-hour plant. This necrosis was

easily observable on the 11th day following inoculation. No necrosis was visible on plants of the other treatments; these showed less general chloronemia on the 11th day than the control plants in the 80-70°F chamber which were by that time in full sporulation.

On the 12th day, a severe chloronemia of inoculated leaves was evident on all plants at 90°F, regardless of treatment. The infected points then appeared as green islands. On the 13th and 14th days, infected chloronemic leaves abscissed. Some 3rd trifoliate leaves with only 12-25 necrotic spots did not become chloronemic and did not abscis. These plants were returned to the 80-70°F chamber where no further symptoms or sporulation developed.

In all 3 temperatures of phase 2, fewer lesions formed on plants which during phase 1 had endured 24 and 32 hours of 90°F.

These results suggested a sensitivity based on the status of development of the host-parasite interaction. Experiments 1 and 2 showed that high temperature prevented development of the parasite without killing it and without any evidence of a protoplasmic response by the host. Development was also stopped at 90°F on plants with symptoms developed beyond the green-white stage (equivalent to 120 hours after inoculation at 80-70°F). Again there was no local protoplasmic response but only general chloronemia and defoliation. In between these periods, however, when heat was endured by reactions in the green-white or even earlier stages, a strong protoplasmic reaction resulted.

The function of experiment 4 was to determine the threshhold for necrotic response. The host-parasite interaction was allowed to proceed to more and more advanced stages before plants were subjected to the heat treatment. One treatment (No. 6 below) was to check on the sterilizing effect of this temperature, reported by Sempio. Another (No. 8) was included to determine if the retarding effect of a cooler environment could be overcome by restoring an environment more conducive to development. There were 3 plants in each of the following treatments:

1. 48 hours at 90°F immediately after penetration period.
2. 24 hours at 80-70°, then 48 hours at 90°, then to 80-70°F.
3. 48 hours at 80-70°, then 90° for 7 days, then to 80-70°F.
4. 72 hours at 80-70°, then to 90°F until end of experiment.
5. 96 hours at 80-70°, then to 90°F until end of experiment.
6. To 90°F immediately following penetration period until 10th day (control).
7. 80-70°F throughout the experiment (control).
8. 70-60° for 72 hours following penetration, then to 80-70°F.

The results of experiment 4 (Fig. 4-A) amplify understanding of those of experiment 3. The controls (treatment 7) moved very rapidly through the stages of development, achieving full sporulation by the 7th

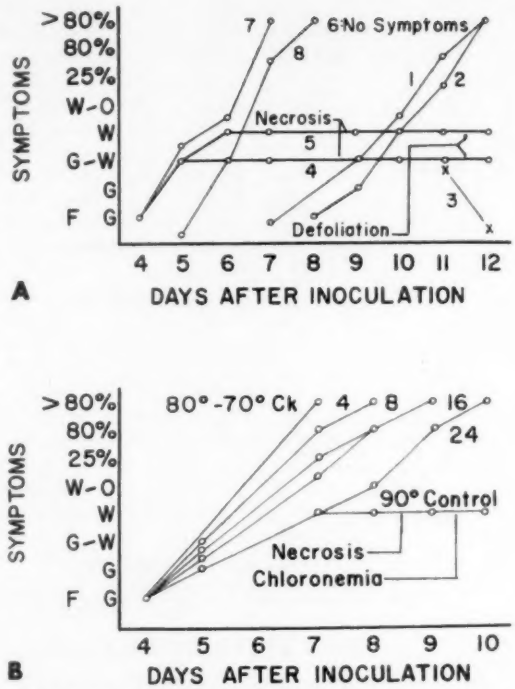


**Fig. 4. A)** Results of experiment 4 showing effects of the various treatments (1-8). **B)** Results of experiment 5 indicating a retardation of symptom development by exposure to 90°F with no necrosis resulting except when plants were continuously at 90° for 7 days.

day after inoculation. Symptoms were retarded on plants of treatment 8 which were held in the 70-60°F environment for 72 hours before being moved to 80-70°.

Plants exposed 48 hours to 90°F immediately after the moisture period then placed at 80-70° (treatment 1) showed a marked time lapse in the 1st symptom expression as well as a slower developmental rate. First symptoms were seen 3 days later than those on control plants and it took twice as long, 6 days as opposed to 3, for full sporulation. In experiment 3, 1st symptoms appeared on the 6th day on plants exposed to 32 hours of 90°F immediately after penetration. In experiment 4, 48 hours of 90° F retarded 1st expression until the 7th day.

Plants of treatment 2 were placed in the 80-70°F environment for 24 hours before being treated identically to treatment 1; results were repeated, except that expression was retarded another 24 hours.

Plants of treatment 3 were in the 80-70°F environment for 48 hours before being subjected to 7 days' treatment at 90°. They were returned to the 80-70°F chamber the day before the necrotic reaction should have occurred. It did not occur. Rather, some green-white punctae were seen on the 11th day; on the 12th, infection loci were visible in transmitted light only. No further development or sporulation occurred, in-

dicating that when the host-parasite interaction was a maximum of 72 hours old when heat was endured, the effect was to limit the parasite without a strong necrotic reaction on the part of the host.

The plants of treatments 4 and 5 showed necrosis and other reactions as seen in experiment 3. These plants had been in the 80-70°F environment for 72 and 96 hours, respectively (host-parasite interaction approximately 96 and 120 hours old), before being subjected to 90°F. It therefore appeared that the threshhold for the local necrosis response lay between 72 and 96 hours under these conditions. The sensitive period disappeared when the interaction was something over 120 hours old. (*Note*: Actual infection followed a germination-penetration period of at least 6 hours.) Less advanced colonization produced smaller local necroses.

Experiment 5 was devised to determine if 4-24-hour exposure to 90°F when the host-parasite interaction was at its most heat-sensitive stage would induce necrosis or otherwise adversely affect the interaction. Three plants were in each treatment.

Experiment 5 (Fig. 4-B) showed that, when exposed to 90°F at its most sensitive time, the host-parasite interaction was adversely affected in proportion to the amount of time it endured the condition. A 4-hour endurance delayed attainment of full sporulation by about 1 day; 8 and 15 hours of exposure caused a 2-day delay; and 24 hours, a 3-day delay. No necrosis occurred after exposures of these lengths. Only those plants left in the 90°F chamber for 5 days showed necrosis. Evidently, exposures to 90°F for times that might be encountered in nature (up to 8 hours) will definitely retard the rate of development of sporulation but will not induce the necrotic reaction.

Discussion.—There were no symptoms when the day-night temperatures were 90-80°F. Infections were present but quiescent during a period of 10 days of these adverse conditions and many could recover fully in a conducive environment. A balance apparently existed between host and parasite activity so that neither the parasite nor the host cells died from parasitic interaction. A constant 90°F for 8 days so weakened the parasite (experiment 4, treatment 3) that it could not compete with the host when optimal temperature was restored. Sempio's experiment demonstrated that a constant 90-93°F for 4 days or 93-97° for 2 days completely sterilized infections. Probably the cooler night temperature of the present study (80°F) rendered the treatment sublethal. How long an infection might remain quiescent is unknown.

Because of the possible epidemiological consequences of this phenomenon, the 3rd experiment was designed to determine whether shorter exposures to high temperature (such as might be encountered in nature) would adversely affect disease expression. The phase 1 results of experiment 3 showed that exposures to 90°F for 8 hours or more definitely retarded symptom expression and reduced severity. When conducive conditions followed this adverse situation, however, "recovery" was normal, that is, symptom progression proceeded as expected (experiment 3, phase 2, 80-70° and 70-60°F). This helps explain difficulties in determining accurately the "field incubation period" of a disease. Obviously, temperature variations of 5-10°F from optimum for periods of 4-8 hours during various times of the host-parasite interaction will retard symptom expression and full sporulation. In nature, such variations are brought about microclimatically by the amounts of foliage, the exposure, wetness, and color of soil; prevailing weather, altitude, and topographic orientation of a field. The field incubation period can vary considerably in different parts of the same field and most certainly in different geographic areas.

The results of experiment 2 further aid our understanding of field observations. Temperature variations between 70° and 80°F had only minor effects on disease expression. But cooler day or night temperature or warmer day temperatures retarded expression. The temperature regime farthest from the optimum (90-60°F) retarded expression the most. A somewhat warmer night (90-70°F) allowed somewhat greater expression, but an even warmer night (90-80°) inhibited expression completely.

These data show the fallacy of using the mean temperature to characterize certain field or laboratory conditions. Were it a good index, the 90-60°, 70-80°, and 80-70°F treatment should have yielded the same results, as would have the 80-60° and 70-70° treatments and the 80-80° and 90-70° treatments.

The discovery and studies of the heat-induced necrotic reaction led to consideration of the host-parasite interaction. In experiment 3, only those leaves with the least developed infections at the beginning of phase 2 (15-, 24-, and 32-hours-of-90°F treatments) showed local necrosis when placed in a 90° environment, whereas more advanced symptoms ceased to develop, later became chlorotic and leaves abscissed, but did not show local necrosis. This suggested that the host-parasite interaction must reach a certain stage before it is so sensitive to the heat stimulus that necrosis occurs. The infection must be less developed than the green-white stage and the earlier experiments indicated that such infections were about 120 hours old at 80-70°F. It was known, however, that even younger infections could (experiment 1) endure 90-80°F for at least 10 days and still resume activity in a favorable environment. Experiment 4 determined the age at which the necrotic reaction began. Infections allowed to develop for only 72 hours (treatment 3) and then moved to 90°F were sterilized with no microscopic necrosis, whereas those which developed for 96 and 120 hours became necrotic.

Experiment 4 did not indicate the exposure to 90° at the most sensitive stage necessary to induce the necrotic reaction. Experiment 5 showed that exposures of greater than 24 hours were necessary, so one would not expect to see this reaction in nature. Nevertheless, high temperatures at this sensitive time will greatly delay the expression of symptoms and sporulation.

The physiologic basis of the heat-induced local necrosis is unknown, but host-parasite interaction does possess a heat-sensitive period. Is this a reflection of imbalance between the competing or complementing respiratory systems of host and parasite? In the earliest stages of infection, 90°F limited the parasite without killing it and without visible protoplasmic response by the host. Later, heat stimulated a hypersensitive, necrotic host response resulting in parasite elimination. When infections were still further developed, however, 90°F only stopped parasite development and host responses were general, not local.

This situation is not yet known to be related to the higher early respiratory activity at infection sites of susceptible hosts followed by a sudden decrease in activity reported by Samborski and Shaw (13), or to the higher initial oxygen uptake of the necrotic fleck reaction than the susceptible uredial reaction as reported by Dickson, *et al.* (2).

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## SPECIES OF LEPTOSPHAERULINA ON FORAGE PLANTS


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SUMMARY


Leaf-inhabiting Ascomycetes characterized by ellipsoid, phragmosporous, or dictyosporous, hyaline to brown ascospores and bitunicate, saccate asci arising individually within the centrum parenchyma of small, spherical, membranous ascocarps are placed in the genus *Leptosphaerulina* (Syn. *Pseudoplea*). Of the 6 species recognized on forage plants in the United States, *L. australis* McAlp., *L. argentinensis* (Speg.) comb. nov., and *L. americana* (Ell. & Ev.) comb. nov. characterized respectively by 4-, 5-, and 6- septate, dictyosporous ascospores are saprobes. The other 3 species are pathogens of legumes. *L. trifolii* (Rost.) Petr. causes pepper spot of *Trifolium* spp. and is potentially capable of producing severe to moderate infection on other legumes, including species of *Medicago*, *Melilotus*, *Pisum*, and *Vigna*. Its low temperature requirement for sporulation limits its occurrence to the cooler periods of the growing season. *L. trifolii* typically produces large, 3-septate, phragmosporous

ascospores and differs from all other species in its slow growth rate in culture. Although *L. **briosiana*** (Poll.) comb. nov. is of importance as the cause of leaf spot of *Medicago* spp., it is potentially capable of producing moderate infection on *Trifolium* spp. and mild infection on other legumes. Because of its slightly higher temperature requirement for sporulation, it differs from *L. trifolii* in seasonal occurrence but it also is a cool-weather pathogen. The dictyosporous ascospores are 4-septate. *L. arachidicola* Yen, Chen & Huang is restricted to *Arachis* spp. It sporulates at temperatures as high as 30°C and causes a leaf spot during the warm summer months. The ascospores, although 4-septate, are smaller than those of *L. **briosiana***, most closely resembling those of *L. australis*. Under suitable conditions on dead leaves or in culture on 20% V-8-juice agar, all species develop mature ascocarps in less than a week. Air-borne ascospores seem the only source of inoculum. The pathogenic species generally require light for ascospore production; the saprobic species may fruit in darkness.

The genus *Leptosphaerulina* includes several fungi associated with leaf diseases of forage plants. These fungi previously have been distributed among various genera, the most familiar of which is *Pseudoplea*. Pepper spot of clovers caused by *L. trifolii* (Rost.) Petr. and leaf spot of alfalfa caused by *P. briosiana* (Poll.) Höhn. are common diseases of forage legumes. Six similar species—*Pleospora americana* Ell. & Ev.; *Pseudoplea gaeumannii* (Müller) Wehm.; *Pleosphaerulina zeicola* Stout; *P. oryzae* Miyake; *Leptosphaerulina vignae* Tehon & Stout; and *L. arachidicola* Yen, Chen & Huang—have been reported on forage plants in the United States but have received little attention. As indicated by the variety of generic combinations, the systematics of these species are in a state of confusion.

To determine the pathogenicity of these fungi on forage plants and to provide a basis for a revision of the classification of fungi of the *Leptosphaerulina* type, morphological, physiological, and pathological studies were carried out on a series of fungus collections and isolations from alfalfa, white clover, and other hosts throughout the eastern United States.

MORPHOLOGY AND TAXONOMY.—On the basis of descriptions of the type species, *Leptosphaerulina australis* McAlp. (21, 48), and the morphological studies of Müller (27) and Wehmeyer (46), the genus *Leptosphaerulina* may be characterized as follows:

LEPTOSPHAERULINA McAlp. 1902

Syn. *Pseudoplea* Höhn. 1918.

Ascocarps small, spherical, parenchymatous, membranous, pale brown, immersed ascostromata erumpent at the apex and opening by a broad pore in a more or less raised collar or short neck of darker brown cells, completely filled with a few large saccate asci. Asci thick-walled, bitunicate, arising individually and successively within the centrum parenchyma, and remaining more or less separated by this parenchymatous tissue at maturity. Ascospores irregularly clustered in the ascus, extremely variable in size, shape, septation, and color; oblong, ellipsoid, or short cylindrical, phragmosporous or muriform, with 3 or more transverse septa and none to several longitudinal septa, surrounded by a thin gelatinous sheath, typically hyaline but often becoming brown. Parasites or saprobes on leaves and herbaceous stems.

The bitunicate asci and the structure of the centrum place *Leptosphaerulina* in the family Pseudosphaeriaceae (11, 19).

Fungi corresponding to the description of *Leptosphaerulina* have been variously placed in the genera *Sphaerulina* (39), *Pleospora* (9, 10, 27, 33), *Catharinia* (40), *Pleosphaerulina* (1, 3, 5, 18, 26, 37, 38, 43, 44), *Saccothecium* (17, 41), *Pseudoplea* (12, 23, 32), and *Pseudosphaeria* (14). *Pleospora* and its hyalosporous segregate *Catharinia* must be excluded from consideration because they are characterized by a pseudoparaphysate centrum, which places them in a different family, the *Pleaosporaceae* (19). *Pleosphaerulina* is an obligate synonym of *Pringsheimia* (12, 47), differing from *Leptosphaerulina* in its ascocarps with thick, dark-colored walls, asci arising in a fascicle from a parenchymatous placenta, clavate ascospores, and occurrence on woody stems. Its centrum characteristics place it in the Dothideaceae (19). Wehmeyer (47) probably correctly considered both *Pleosphaerulina* and *Pringsheimia* synonyms of *Saccothecium*, but a different interpretation of the type species (13, 28, 34) would support the genus *Pringsheimia* and make *Saccothecium* and *Massaria* synonymous. In any event, *Saccothecium* is not applicable for fungi of the *Leptosphaerulina* type. The application of the name *Sphaerulina* is so doubtful that it should be rejected or possibly considered a synonym of *Pringsheimia* (*Saccothecium*), if *Sphaerulina intermixta* (Berk. & Br.) Sacc. is accepted as the type species (47). Von Höhnel (13), however, based the genus on *Spaerulina myriades* (DC) Sacc. and employed *Sphaerulina* for fungi similar to *S. rubi* Demaree & Wilcox (8), which are distinguished from *Mycosphaerella* spp. only by their phragmosporous ascospores (14).

*Pseudoplea* has been separated from *Leptosphaerulina* on the basis of its hyaline ascospores; the ascospores of *Leptosphaerulina* have been described as ultimately becoming brown (21). Ascospore color, however, is variable, and the ascospores of species in both genera may ultimately become brown. Consequently, Müller (29) and Wehmeyer (48) considered *Pseudoplea* a synonym of *Leptosphaerulina*. *Wettsteinina* (Syn. *Pseudosphaeria*) differs from *Leptosphaerulina* in its larger, consistently phragmosporous ascospores with more conspicuous gelatinous sheaths and tardy formation of septa (35). As noted by Wehmeyer (48), species of these 2 genera intergrade; and probably, comparative studies will demonstrate that *Wettsteinina* also is synonymous with *Leptosphaerulina*.

**Fig. 1-17.** Ascospores and asci of *Leptosphaerulina* spp. 1-4) *L. trifolii*: 1) No. 942, from leaves of *Trifolium pratense*, Ga. 2) No. 949, from leaves of *T. repens*, Ga. 3) No. 943, from leaves of *T. incarnatum*, Ga. 4) No. 798, (*T. repens*, W. Va.) from culture. 5-8) *L. briosiana*. 5) No. 947, from leaves of *Medicago hispida*, Ga. 6) No. 948, from leaves of *M. sativa* Ga. 7) No. 927, (*M. hispida*, Ga.) from culture. 8) No. 797, (*M. sativa* W. Va.) from culture. 9-11) *L. australis*. 9) From type collection of *Pleosphaerulina zeicola* on *Zea mays*, Ill. 10) No. 1327, (*T. repens*, Ga.) from culture. 11) No. 275, (grass leaf, Pa.) from culture. 12-13) *L. arachidicola*. 12) No. 1337, (*Arachis hypogaea*, Taiwan) from culture. 13) No. 1325, (*A. hypogaea*, Ga.) from culture. 14-15) *L. argentinensis*. 14) From type collection of *Pleosphaerulina argentinensis* on *Datura*, Argentina. 15) No. 442, (*Melilotus alba*, Pa.) from culture. 16-17) *L. americana*. 16) From type collection of *Pleospora americana* on *Vicia sativa*, Miss. 17) No. 943, (*T. pratense*, Ga.) from culture.

In all collections and isolations included in this study, the ascocarps were similar in structure and fitted the generic description given for *Leptosphaerulina*. A characteristic feature of all species was the peculiar structure of the apical wall of the ascus. The inner wall was especially thick at the apex and was partially penetrated by a pore into which the protoplast extended. Beneath its dome-shaped apex, the pore was extended laterally into a flat, annual ring resembling the brim of a hat (Fig. 5, 17). This characteristic structure was illustrated by Bubák (4). Primarily on the basis of size and septation of the ascospores and of colony characteristics in culture, the collections were divided into 6 groups to which the most appropriate available specific names were applied. Data on ascospore characteristics of representative collections are in Table 1 and Fig. 1-17. Whenever possible, observations were made both on material collected in the field and on isolates from these collections grown on 20% V-8-juice agar plates under fluorescent light in an incubator at 20°C. Ascospore measurements were made on samples of 25 spores.

1) LEPTOSPHAERULINA AUSTRALIS McAlp. 1902

Syn. *Pleosphaerulina oryzae* Miyake 1910
*Leptosphaerulina vignae* Tehon & Stout 1928
*Pleosphaerulina setariae* Lobik 1928
*Pleosphaerulina zeicola* Stout 1930
*Pleospora gaeumannii* Müller 1951
*Pseudoplea gaeumannii* (Müller) Wehm. 1955

On hosts, ascocarps 40-170 $\mu$ diameter; asci 50-90 x 30-45 $\mu$; ascospores 25-41 x 10-15 $\mu$, means 32.4-35.3 x 12.0-14.0 $\mu$, 3-6 x 1-3-septate, means 3.6-4.0 x 1.8-2.3 septa, 100% muriform. Colonies of fast-growing, dark, appressed mycelium covered with small, dispersed ascocarps; ascocarps 69-188 $\mu$ diameter; asci 65-91 x 33-55 $\mu$; ascospores 29-41 x 11-15 $\mu$, means 33.0-37.7 x 12.6-13.9 $\mu$, 3-5 x 0-4-septate, means 3.9-4.1 x 1.7-2.3 septa, 96-100% muriform.

On dead leaves of unidentified grass, *Poa annua* L., and *Trifolium repens* L. (killed by *Rhizoctonia solani* Kühn) and on seed of *Panicum fasciculatum* Swartz. in a germinator, from Georgia and Pennsylvania.

The fungus in these collections is identical with that on grass culms described by Müller (27) as *Pleospora gaeumannii*. This species was transferred to *Pseudoplea* by Wehmeyer (46) who reported it on *Agrostis*, *Poa*, *Rosa*, and *Ligustrum* and as a culture contaminant. A number of similar fungi, however, have been described under names that antedate *P. gaeumannii*. The earliest of these is *Leptosphaerulina australis* described by McAlpine (21) from leaves of *Prunus armeniaca* L. as with globose, membranous, parenchymatous, pale-brown ascocarps averaging 150 $\mu$ in diameter; saccate asci measuring 75-80 x 28-50 $\mu$; and oblong ascospores, at first hyaline but ultimately brown, 5-septate usually with 2 longitudinal septa, and measuring 30-32 x 11 $\mu$. McAlpine's figures show both 3- and 5-septate ascospores. McAlpine (21) reported this species also on leaves of *Vitis*, *Dolichos*, *Poa*, and *Lolium*. Although the type specimen could not be located, collections on *Dolichos*, *Humulus*, *Aquilegia*, and *Papaver* were received from S. Fish, Chief Biologist, Department of Agriculture, Victoria, Australia. In the collection on *Dolichos* (G. H. R., July 1901, Armadale), which is probably 1 of the original collections mentioned by McAlpine (21), a few ascospores resembling those illustrated by McAlpine were found. These measured 30-37 x 12-15 $\mu$, were 4-5 x 2-4-septate, and were hyaline although they appeared fully mature. In these same collections, Wehmeyer (48) found a few ascocarps 50-100 $\mu$ in diameter containing large saccate asci measuring 53-70 x 35-37 $\mu$. Mature ascospores were hyaline, 3-4 x 2-septate, and 27-32 x 12.5-14 $\mu$. Although the extant material of *L. australis* is disappointing, it indicates the probable identity of our collections with this species.

The type collections of *Pleosphaerulina zeicola* (44) and *Leptosphaerulina vignae* (45) were made available by J. C. Carter, Illinois State Natural History Survey. In the type of *P. zeicola* (No. 21182, G. L. Stout, October 26, 1927, *Zea mays* L., Highland, Madison County, Ill.), ascocarps were 138-179 $\mu$ in diameter. Ascospores were hyaline, 27-35 x 11-14 $\mu$, mean 31.4 x 12.3 $\mu$, 3-5 x 1-4-septate, means 4.5 x 2.3 septa, and 100% muriform. In the type of *L. vignae* (No. 20937, G. L. Stout, September 5, 1927, Huey, Clinton County, Ill.), no ascocarps could be found. On a prepared slide accompanying the specimen were a few ascocarps 98-129 $\mu$ in diameter. Ascospores within the asci were hyaline, but the free ascospores were moderately dark brown, 29-33 x 12-14 $\mu$, and 3-5 x 2-3-septate. These species are considered synonymous with *L. australis*. Although the type collections could not be obtained, the descriptions and illustrations of *Pleosphaerulina oryzae* (26) and *P. setariae* (18) indicate the relationship of these species to *L. australis*.

2) LEPTOSPHAERULINA ARACHIDICOLA Yen, Chen & Huang 1956

Ascocarps 64-140 $\mu$ diameter; asci 53-87 x 28-42 $\mu$; ascospores 23-40 x 11-17 $\mu$, means 28.8-31.0 x 12.3-

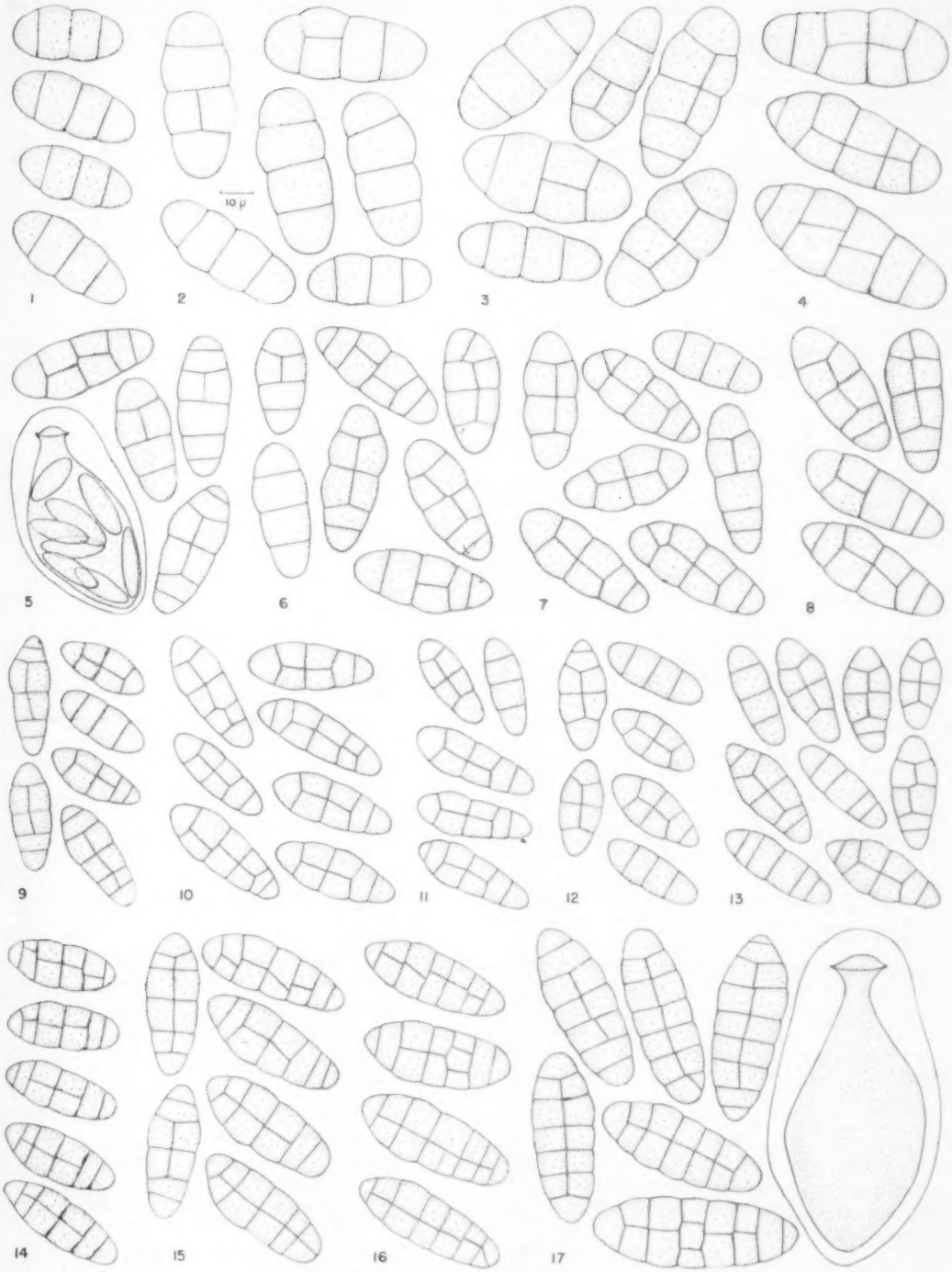
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TABLE 1. Dimensions and septation of ascospores of *Leptosphaerulina* spp. on their hosts and in culture on V-8-juice agar

| Species and collection | Source | Length x width ($\mu$) | Mean ($\mu$) | No. of septa | Mean | Muriform (%) |
|---|---|---|---|---|---|---|
| *L. trifolii:* | | | | | | |
| 926, *Trifolium repens* (Ga.) | host | 28-39 x 11-17 | 33.7 x 14.4 | 3 x 0-1 | 3.0 x 0 | 4 |
| | cult. | 43-55 x 18-24 | 49.0 x 20.6 | 3-4 x 0-2 | 3.2 x 1.0 | 72 |
| 934, *T. incarnatum* (Ga.) | host | 36-48 x 15-21 | 40.3 x 17.6 | 3-4 x 0-2 | 3.1 x 1.0 | 80 |
| | cult. | 43-61 x 20-26 | 51.3 x 22.2 | 3-5 x 0-2 | 3.6 x 0.9 | 68 |
| 942, *T. pratense* (Ga.) | host | 25-34 x 12-15 | 29.2 x 13.4 | 3 x 0 | 3.0 x 0 | 0 |
| | cult. | 38-56 x 17-21 | 46.5 x 18.2 | 3-4 x 0-2 | 3.1 x 0.4 | 40 |
| 949, *T. repens* (Ga.) | host | 35-49 x 15-21 | 43.3 x 18.5 | 3 x 0-1 | 3.0 x 0 | 4 |
| | cult. | 44-56 x 20-24 | 49.9 x 20.7 | 3-4 x 0-2 | 3.4 x 1.0 | 64 |
| 798, *T. repens* (W. Va.) | cult. | 50-62 x 20-24 | 54.2 x 22.3 | 4-5 x 1-4 | 4.4 x 2.0 | 100 |
| 682, *T. pratense* (Maine) | cult. | 46-58 x 19-24 | 52.5 x 21.3 | 3-5 x 0-3 | 4.5 x 1.7 | 88 |
| *L. briosiana:* | | | | | | |
| 925, *Medicago sativa* (Ga.) | host | 26-36 x 11-16 | 32.2 x 14.3 | 3-4 x 0-2 | 3.1 x 1.0 | 72 |
| | cult. | 29-44 x 11-17 | 38.1 x 16.1 | 3-4 x 0-2 | 3.6 x 1.3 | 88 |
| 927, *M. hispida* (Ga.) | host | 25-38 x 11-15 | 31.4 x 13.2 | 3-4 x 0-3 | 3.6 x 1.4 | 92 |
| | cult. | 26-49 x 11-18 | 42.4 x 16.4 | 3-5 x 0-4 | 4.1 x 1.8 | 88 |
| 948, *M. sativa* (Ga.) | host | 30-43 x 14-18 | 37.8 x 16.0 | 3-5 x 1-2 | 3.6 x 1.7 | 100 |
| | cult. | 35-47 x 14-18 | 41.0 x 16.7 | 3-5 x 0-2 | 4.2 x 1.4 | 96 |
| 952, *M. lupulina* (Ga.) | host | 30-43 x 14-18 | 39.1 x 16.0 | 3-4 x 0-2 | 3.6 x 1.2 | 80 |
| | cult. | 33-47 x 14-17 | 40.9 x 16.7 | 4-5 x 0-3 | 4.5 x 1.7 | 96 |
| 953, *T. isthmocarpum* (Ga.) | host | 32-41 x 14-17 | 37.3 x 15.8 | 3-5 x 0-2 | 3.2 x 1.6 | 92 |
| | cult. | 38-50 x 14-19 | 43.3 x 16.8 | 4-5 x 0-3 | 4.4 x 1.6 | 96 |
| 797, *M. sativa* (W. Va.) | cult. | 40-49 x 16-20 | 44.0 x 17.5 | 4-5 x 0-4 | 4.5 x 1.7 | 96 |
| *L. australis:* | | | | | | |
| 1101, *Panicum fasciculatum* (Ga.) | host | 25-37 x 10-13 | 32.4 x 12.0 | 3-4 x 1-3 | 3.6 x 1.8 | 100 |
| | cult. | 32-41 x 12-15 | 37.7 x 13.9 | 4-5 x 1-4 | 4.1 x 2.3 | 100 |
| 275, Grass (Pa.) | cult. | 29-40 x 11-13 | 33.0 x 12.0 | 3-5 x 0-2 | 3.9 x 1.7 | 96 |
| 1326, *Poa annua* (Ga.) | host | 32-41 x 13-15 | 35.3 x 14.0 | 3-5 x 2-3 | 4.0 x 2.3 | 100 |
| | cult. | 33-40 x 12-15 | 37.3 x 13.3 | 4-5 x 1-3 | 4.0 x 2.2 | 100 |
| 1327, *T. repens* (Ga.) | host | 30-41 x 12-15 | 34.8 x 13.9 | 3-6 x 1-3 | 4.0 x 2.2 | 100 |
| | cult. | 33-41 x 12-14 | 37.0 x 13.2 | 4-5 x 1-3 | 4.0 x 2.0 | 100 |
| *L. arachidicola:* | | | | | | |
| 1325, *Arachis hypogaea* (Ga.) | host | 26-35 x 10-14 | 31.0 x 12.4 | 3-5 x 0-2 | 4.1 x 1.3 | 64 |
| | cult. | 27-35 x 11-15 | 32.5 x 13.5 | 3-5 x 0-3 | 4.4 x 1.7 | 84 |
| 1356, *A. hypogaea* (Ga.) | host | 23-33 x 10-15 | 28.9 x 12.6 | 3-5 x 0-2 | 3.8 x 1.7 | 84 |
| | cult. | 29-35 x 11-17 | 32.5 x 13.2 | 3-5 x 0-2 | 4.2 x 1.6 | 80 |
| *L. argentinensis:* | | | | | | |
| 1412, *Pueraria lobata* | host | 24-30 x 12-14 | 28.1 x 12.4 | 3-5 x 1-3 | 4.2 x 2.1 | 100 |
| | cult. | 32-40 x 13-16 | 36.4 x 14.8 | 3-5 x 1-4 | 4.9 x 2.6 | 100 |
| 442, *Melilotus alba* (Pa.) | host | 33-41 x 14-17 | 37.3 x 15.1 | 4-6 x 1-4 | 4.9 x 2.9 | 100 |
| | cult. | 35-43 x 14-17 | 39.1 x 15.4 | 5-6 x 2-5 | 5.2 x 3.3 | 100 |
| 943, *T. pratense* (Ga.) | host | 35-45 x 13-17 | 39.0 x 14.8 | 5-6 x 2-5 | 5.7 x 4.0 | 100 |
| | cult. | 44-58 x 15-20 | 49.9 x 17.8 | 6-8 x 3-6 | 6.1 x 4.8 | 100 |
| 863, *Phleum pratense* (Pa.) | host | 43-49 x 15-18 | 44.8 x 16.1 | 5-6 x 3-4 | 5.6 x 3.7 | 100 |
| | cult. | 44-59 x 17-21 | 51.1 x 18.6 | 6-8 x 3-6 | 6.3 x 5.0 | 100 |

12.9 μ, 3.5 x 0-2-septate, mean 3.6-4.1 x 1.3-1.7 septa, 64-84% muriform. Colonies in culture of fast-growing, dark, appressed mycelium covered with small, dispersed ascocarps; ascocarps 90-216 μ diameter; asci 53-101 x 31-42 μ; ascospores 26-38 x 10-17 μ, means 29.6-32.5 x 12.6-13.5 μ, 3-5 x 0-3-septate, means 3.7-4.4 x 1.4-1.7 septa, 68-84% muriform.

On diseased leaves of *Arachis hypogaea* L. and *A. monticola* Krapov. & Rigoni from Georgia and Virginia.

Specimens of *Leptosphaerulina arachidicola* received from K. T. Huang, Provincial College of Agriculture, Taichung, Taiwan, contained only immature ascospores, but an accompanying isolate of the fungus produced colonies identical with those of the United States isolates with ascocarps 124-248 μ in diameter, asci 56-92 x 31-39 μ, and hyaline ascospores 26-33 x 10-14 μ, mean 29.6 x 12.6 μ, 3-4 x 0-2-septate, mean 3.2 x 1.6 septa, and 80% muriform. Although our collections differ from the Taiwan material, having slightly larger ascospores with a greater number of septa, they agree in other respects with the isolate from Taiwan and with the description of *L. arachidicola* (49) and are referred to this species.

*Leptosphaerulina arachidicola* corresponds closely with *L. australis* in the production of relatively small, typically 4-septate ascospores, and only minor differences can be found between the 2 species. The most useful morphological feature distinguishing *L. arachidicola* is its dimorphic ascospores. The majority of the ascospores are muriform and ellipsoid, but up to 34% are phragmosporous and cylindrical. Few or no phragmosporous ascospores appear among those discharged by the asci on agar plates, whereas crushed mounts of the same material show an abundance of such spores that are fully mature. Both types occur in the same ascus. Although there is no constant ratio, as many as half of the spores in an individual ascus may be of the cylindrical, phragmosporous type. Muriform ascospores of *L. arachidicola* (Fig. 12, 13) present a stubbier appearance when compared with those of *L. australis* (Fig. 9-11), but spore measurements revealed only slight differences. Muriform ascospores of *L. arachidicola*, with mean dimensions of 31.2 x 13.4 μ, had a length/width ratio of 2.3; whereas those of *L. australis* with mean dimensions of 34.9 x 13.2 μ, had a length/width ratio of 2.6. *L. arachidicola* differs also in its association with a leaf disease of *Arachis* spp. (20, 49).

3) **LEPTOSPHAERULINA BRIOSIANA** (Poll.) comb. nov.

Syn. *Pleosphaerulina briosiana* Poll. 1902
*Pleosphaerulina briosiana* var. *brasiliensis* Putt. 1905
*Pseudoplea briosiana* (Poll.) Höhn. 1918
*Pseudoplea medicaginis* Miles 1925
*Pleosphaerulina briosiana* var. *macrospora* Camara & Luz 1941

Ascocarps 83-152 μ diameter; asci 53-98 x 31-48 μ; ascospores 26-46 x 11-18 μ, means 32.2-39.7 x 14.3-16.2 μ, 3-5 x 0-2-septate, means 3.1-3.8 x 1.0-1.7 septa, 72-100% muriform. In culture producing fast-growing colonies of appressed, dark mycelium covered with small, dispersed ascocarps; ascocarps 83-276 μ diameter; asci 109-122 x 55-59 μ; ascospores 26-50 x 11-20 μ, means 38.1-42.4. x 16.1-16.8 μ, 3-5 x 0-4-septate, means 3.6-4.5 x 1.3-1.8 septa, 88-96% muriform.

On diseased leaves of *Medicago sativa* L., *M. hispida* Gaertn., *M. lupulina* L., *Trifolium hybridum* L.; and *T. isthmocarpum* Brot. from Georgia, Maine, Pennsylvania, and West Virginia.

*Pseudoplea briosiana* is generally used for fungi of this type on *Medicago* species and, with the new combination in *Leptosphaerulina*, is so applied here. There is little, however, in the earlier descriptions on which to base the name except its occurrence on *Medicago* spp. and production of consistently muriform ascospores. The original description of *Pleosphaerulina briosiana* by Pollacci (37) from material on *M. sativa* and *M. falcata* L. in Italy applies to our collections except for the much smaller dimensions of the ascospores which were reported to be 20-25 x 6-8 μ and 3-4 x 1-2-septate. Puttemans (38) described a similar fungus on *M. sativa* in Brazil with 3-septate, muriform ascospores measuring 25-30 x 10-12 μ as *Pleosphaerulina briosiana* var. *brasiliensis*. After an examination of Pollacci's type collection, Bubák (4) stated that the smaller ascospore dimensions given by Pollacci were in error. He therefore referred his Austrian collection on *M. sativa* with 3- x 2-septate ascospores measuring 32-44 x 16-21 μ to *P. briosiana*. Ascospore dimensions recorded by Bubák seem unusually large, but they are approximated by those for our culture No. 797 (Table 1). Unusual also is the constant production of 3-septate ascospores reported by Bubák (4) and Puttemans (38). In our culture No. 925, however, 76% of the ascospores were 3-septate (Table 1). Miles' (23) description of *Pseudoplea medicaginis* on *M. hispida* with 3-4 x 1-2-septate ascospores measuring 28-39 x 10-16 μ (mean 35 x 13.5 μ) agrees almost exactly with our collection No. 927 on the same host, and this cannot be distinguished from collections No. 925 and No. 948 on *M. sativa* (Table 1).

*Leptosphaerulina briosiana*, with chiefly 4-septate, muriform ascospores, differs from *L. australis* and *L. arachidicola* in its larger ascospores and asci and in its regular occurrence on diseased leaves of *Medicago* spp. Its occurrence on *Trifolium* spp. is unusual. Two of the isolates from *Trifolium* spp. examined in this study, however, have been referred to *L. briosiana*. *Pleoshaerulina brisosiana* var. *macrospora* described (5) on *T. incarnatum* L. in Portugal as with typically 4-septate ascospores measuring 34-46 x 16-17 μ is, therefore, tentatively placed under *L. briosiana* although the type collection could not be located.

4) LEPTOSPHAERULINA TRIFOLII (Rost.) Petr.

Syn. *Sphaerulina trifolii* Rost. 1899
*Pseudoplea trifolii* (Rost.) Petr. 1921
*Pleospora trifolii* (Rost.) Petr. 1927
*Pseudosphaeria trifolii* (Rost.) Höhn. 1929

*Saccothecium trifolii* (Rost.) Kirsch. 1938

Ascocarps 124-207 $\mu$ diameter; asci 62-95 x 42-59 $\mu$; ascospores 25-49 x 11-21 $\mu$, means 29.2-43.3 x 13.4-18.5 $\mu$, 3-4 x 0-2-septate, means 3.0-3.1 x 0-1 septa, 0-80% muriform. Colonies in culture slow-growing, covered with a dense black crust of large, often compound ascocarps; ascocarps 152-276 $\mu$ diameter, when compound 524-580 x 276-414 $\mu$; asci 91-137 x 65-71 $\mu$; ascospores 38-62 x 17-26 $\mu$, means 46.5-54.2 x 18.2-22.3 $\mu$, 3-5 x 0-4-septate, means 3.1-4.5 x 0.4-2 septa, 40-100% muriform.

On diseased leaves of *Trifolium repens*, *T. pratense* L., and *T. incarnatum* from Georgia, Maine, Pennsylvania, and West Virginia.

Rostrup's (39) original description of *Sphaerulina trifolii* on *Trifolium repens* in Denmark is essentially in agreement with this concept of *Leptosphaerulina trifolii*, the 3-septate, phragmosporous ascospores being typical of the species and leading von Höhnel (14) to place it in *Pseudosphaeria* (*Wettsteinina*). Petrak (36) also recently suggested the new combination *L. trifolii* (Rost.) Petr. Although the ascospore dimensions given by Rostrup are unusually small (32-33 x 12-15 $\mu$), they are nearly the same as those for our collection No. 942 which in culture produced ascospores of typical size (Table 1). Collection No. 934 on *T. incarnatum* (Table 1) is a somewhat aberrant form with more frequently septate, muriform ascospores. Isolates from *T. repens*, however, may show as high a percentage of muriform ascospores in culture. Also, the larger dimensions of the ascospores and the colony characteristics in culture indicate a relationship with *L. trifolii*. Probably, cultural studies of the fungus on *T. incarnatum* in Portugal described as *Pleosphaerulina briosiana* var. *macrospora* (5) would demonstrate its relationship with *L. trifolii* rather than with *L. briosiana*.

Collections included under *Leptosphaerulina trifolii* differ from those referred to *L. briosiana* in a combination of characters which, when definitely expressed, clearly distinguish the 2 species but, when weakly expressed, exhibit a degree of overlapping between the 2 groups. The ascocarps and asci of *L. trifolii* are larger, particularly in culture. The ascospores are much larger when developed under favorable conditions in the field and in culture. They tend to be 3-septate with a smaller number of longitudinal septa and a smaller percentage of muriform spores (often nearly all phragmosporous), whereas the ascospores of *L. briosiana* tend to be 4-septate and are typically muriform. *L. trifolii* is distinct from all other species in colony characteristics and also in its association with leaf spot of *Trifolium* species.

5) **LEPTOSPHAERULINA AMERICANA** (Ell. & Ev.) comb. nov.

*Pleospora hyalospora* Ell. & Ev. 1890 non Speg. 1879
Syn. *Pleospora americana* Ell. & Ev. 1892
*Catharinia americana* (Ell. & Ev.) Sacc. 1895
*Pleosphaerulina americana* (Ell. & Ev.) Berl. 1900
*Pleosphaerulina hyalospora* Berl. 1900

*Pleosphaerulina ellisi* Speg. 1909

Ascocarps 126-140 $\mu$ diameter; asci 101-106 x 45-48 $\mu$; ascospores 34-49 x 13-18 $\mu$, means 39.0-44.8 x 14.8-16.1 $\mu$, 5-6 x 2-5-septate, means 5.6-5.7 x 3.7-4.0, 100% muriform. Colonies fast-growing with abundant pale gray aerial mycelium and sparse production of ascocarps or with little aerial mycelium and numerous ascocarps dispersed on an appressed mycelium; ascocarps 168-331 $\mu$ diameter; asci 87-140 x 36-70 $\mu$; ascospores 44-59 x 15-21 $\mu$, means 49.9-51.1 x 17.8-18.6 $\mu$, 6-8 x 3-6-septate, means 6.1-6.3 x 4.8-5.0 septa, 100% muriform.

On dead leaves of *Trifolium pratense* and *Phleum pratense* L. from Georgia and Pennsylvania.

A similar fungus was described by Ellis and Everhart (9) as *Pleospora hyalospora*. This combination was illegitimate since it is a homonym of *Pleospora hyalospora* Speg. Ellis and Everhart (10), therefore, renamed their fungus *Pleospora americana* which is the first valid name for this species. This species was based on 2 collections: 1) S. M. Tracy, May 24, 1890, *Vicia sativa* L., Starkville, Miss.; and 2) S. M. Tracy, June 7, 1890, *Pisum sativum* L., Starkville, Miss. These cotype collections were obtained from the New York Botanical Garden through C. T. Rogerson. In the collection on *Vicia*, the ascocarps were 70-182 $\mu$ in diameter; asci 70-75 x 41-52 $\mu$; and fully mature free ascospores were 40-50 x 17-20 $\mu$, means 44.4 x 17.4 $\mu$, 6 x 3-6-septate, mean 6 x 4.9 septa, and 100% muriform. Ascospores within the asci were smaller and usually 5 x 1-2-septate. In the collection on *Pisum*, ascocarps were 84-160 $\mu$ in diameter and asci 84-91 x 40-56 $\mu$. Most of this material appeared immature, and the ascospores within the asci were 5 x 1-2-septate. The few free ascospores were 37-46 x 14-18 $\mu$ and 6 x 3-4-septate. Berlese (3) considered these 2 collections distinct species, basing the new combination *Pleosphaerulina americana* on the *Pisum* collection with 5-septate ascospores and the new species *Pleosphaerulina hyalospora* on the *Vicia* collection with 6-septate ascospores. The difference in septation between the 2 collections apparently represents merely a difference in maturity since, although the majority of the ascospores in the *Pisum* collection were within the asci and were 5-septate, the few free ascospores were 6-septate as were the mature ascospores in the *Vicia* collection. Consequently, these 2 collections are considered a single species which is transferred to the genus *Leptosphaerulina* as *L. americana*.

*Leptosphaerulina americana* differs from all other species in that the ascospores are almost constantly 6-septate and are provided with 4-5 longitudinal septa. The ascospores also are much larger than those of any other species except *L. trifolii*. It has now been found as a saprobe on leaves of *Vicia*, *Pisum*, *Trifolium*, and *Phleum*. A previous report of this species on *Trifolium* by Ellis and Everhart (10) apparently was in error. Two specimens labeled *Pleospora americana* on *T. repens* were found in the Ellis Collection in the New York Botanical Garden Herbarium; J. Dearness, Au-

gust 1889, London, Canada; and J. Dearness, August 1890, London, Canada. Only *Polythrincium* and *Stagonospora* were present in the 1890 collection. In the 1889 collection, there was, in addition, a trace of a *Leptosphaerulina* with 3 x 0-septate ascospores measuring 33-40 x 15-17 $\mu$. This was *L. trifolii*.

6) **LEPTOSPHAERULINA ARGENTINENSIS** (Speg.) comb. nov.

Syn. *Pleosphaerulina argentinensis* Speg. 1909
*Pleosphaerulina smilacicola* Speg. 1909

Ascocarps 104-202 $\mu$ diameter; asci 78-92 x 39-45 $\mu$; ascospores 24-41 x 12-17 $\mu$, means 28.1-37.3 x 12.4-15.1 $\mu$, 3-6 x 1-4-septate, means 4.2-4.9 x 2.1-2.9 septa, 100% muriform. In culture forming fast-growing colonies of dark mycelium densely covered with dispersed ascocarps; ascocarps 70-213 $\mu$ diameter; asci 76-104 x 39-50 $\mu$; ascospores 32-43 x 13-17 $\mu$, means 36.4-39.1 x 14.8-15.4 $\mu$, 3-6 x 1-5-septate, means 4.9-5.2 x 2.6-3.3 septa, 100% muriform.

On dead leaves of *Melilotus alba* Desr., *Pueraria lobata* (Willd.) Ohwi, and *Sorghum halepense* (L.) Pers. from Pennsylvania and Georgia.

These collections were compared with the type (C. Spegazzini, No. 2205, May 1905, *Datura stramonium* L., Buenos Aires, Argentina) of *Pleosphaerulina argentinensis* (43) obtained from the Museo Instituto Spegazzini, Universidad de la Plata, courtesy of J. C. Lindquist. Ascocarps produced in the dead margins of the *Datura* leaf were spherical, immersed, yellowish-brown, and 97-152 $\mu$ in diameter. They contained a few large, saccate, thick-walled asci measuring 61-85 x 33-38 $\mu$. The ascospores, which appeared slightly immature, were ellipsoid, hyaline, 30-37 x 13-15 $\mu$, mean 34.3 x 13.7 $\mu$, and 5 x 2-4-septate, mean 5 x 2.8 septa. Our collections appeared essentially similar to the type of *P. argentinensis*, which belongs in *Leptosphaerulina*. *Pleosphaerulina smilacicola* also is characterized by 5-septate ascospores, and nothing in the description of this species (43) distinguishes it from *L. argentinensis*. The *Pleospora* sp. on leaves of *Ricinus communis* L., described by Cook (7) as having ascocarps 122-144 $\mu$ in diameter and 4-5 x 2-3-septate ascospores measuring 36 x 15 $\mu$, may represent *L. argentinensis* if the ascospore dimensions given approximate the mean. The account of this fungus, however, is too meager to exclude the possibility that it should be referred to *L. australis*.

*Leptosphaerulina argentinensis* is separated from *L. americana*, to which Spegazzini (43) considered it related, because the ascospores are smaller and are most frequently 5-septate rather than 6-septate. Actually, it appears more closely related to *L. australis* from which it differs in its larger, 5-septate ascospores. *L. vitrea* (Rostr.) Woronich., another species with 5-septate ascospores, differs from *L. argentinensis* in that the ascospores are much smaller (21-26 x 9-11 $\mu$) and are more asymmetrical with a swollen, globose anterior portion (29). *L. vitrea* undoubtedly is a synonym of *L. pulchra* (Wint.) Barr (2).

CONIDIAL STAGE.—On the basis of association on the host, *Stagonospora meliloti* (Lasch) Petr. (32) and *Alternaria* sp. (42) have been suggested as imperfect stages of *Leptosphaerulina trifolii* and *L. briosiana*, but no connection between these fungi has been proved experimentally. Nelson and Kernkamp (31) reported that ascocarps of *L. briosiana* developed in single-conidium cultures of *Stemphylium botryosum* Wallr. and conidia in single-ascospore cultures of *L. briosiana* from *Medicago sativa*. Subsequently, in describing the infection chain of *Stemphylium* leaf spot of *M. sativa*, Nelson (30) stated that several cycles of infection with ascospores of *L. briosiana* occurred from early spring through June; whereas the conidial stage, *S. botryosum*, was predominant from mid-July until early fall, when perithecial initials of *L. briosiana* again formed in the old lesions. Allison, *et al.* (1) confirmed the connection between *S. botryosum* and *Pleospora herbarum* (Pers. ex Fr.) Rab. which has been established for many years but reported that "*Stemphylium*-type" conidia were produced very sparingly in cultures of all isolates of *Leptosphaerulina* from *M. sativa* and *Trifolium repens*. McDonald (22) was unable to establish any connection between *Leptosphaerulina* and *Stemphylium*.

The present study failed to demonstrate the occurrence of a conidial stage in any *Leptosphaerulina* sp. Diseased leaves of *M. sativa* collected in the field often yielded both *L. briosiana* and *S. botryosum* from the same leaflet. Isolates from ascospores of *L. briosiana* produced only ascocarps of *L. briosiana*. Isolates from conidia of *S. botryosum* produced similar conidia in culture and, after 3 months, ascocarps of *Pleospora herbarum*. In a single greenhouse test in which *M. sativa* and *T. repens* plants were inoculated with *L. briosiana* and *L. trifolii*, both *Leptosphaerulina* ascocarps and *Stemphylium* conidia developed on the leaves. Contamination was indicated since *Stemphylium* occurred on some of the control plants and also since the species on *M. sativa* was *S. botryosum* and that on *T. repens* was *S. trifolii* Graham. In repeated tests in which contamination was excluded, only *Leptosphaerulina* ascocarps developed on leaves inoculated with ascospores of *Leptosphaerulina* spp.

THE DISEASES.—Diseases referred to as *Leptosphaerulina* (*Pseudoplea*) leaf spot, or pepper spot, have been reported on various legumes throughout the world since the latter part of the nineteenth century. Pepper spot caused by *L. trifolii* is moderately severe each year in the eastern United States on *Trifolium repens* (Ladino white clover) and probably contributes to its lack of persistence. In the Northeast, pepper spot is most prevalent in early spring with a lesser build-up again in the fall, whereas in the South it is essentially a midwinter disease and gradually disappears during the warmer months of spring until only traces remain in early June. The common symptoms are numerous tiny, black, sunken lesions on leaves and petioles; although under favorable conditions, spots up to 3 mm in diameter may develop on leaves of very susceptible plants. Severely infected leaves wither and turn brown.

TABLE 2. Mycelial growth of 6 *Leptosphaerulina* spp. on V-8-juice agar after 5 days at 20°C

| Species | No. of isolates | Colony diameter (mm) | | Radial growth/ day (mm) |
|---|---|---|---|---|
| | | Range of isolates[a] | Avg. | |
| *L. trifolii* | 10 | 10-19 | 13.4 | 2.1 |
| *L. briosiana* | 9 | 23-30 | 26.1 | 4.6 |
| *L. arachidicola* | 6 | 28-30 | 29.2 | 5.2 |
| *L. americana* | 2 | 31-33 | 32.0 | 5.8 |
| *L. argentinensis* | 3 | 30-34 | 32.3 | 5.9 |
| *L. australis* | 4 | 31-36 | 33.8 | 6.2 |

[a] Average of 3-5 replications for each isolate.

Similar, but generally less severe symptoms are produced on other *Trifolium* spp.

Leaf spot of *Medicago sativa* (alfalfa) caused by *L. briosiana* has been recognized for many years but has been considered of minor importance. During the cool, wet growing seasons of 1956, 1958, and 1960 in the northeastern United States, however, this disease reached epidemic proportions and was responsible for considerable defoliation, particularly on the aftermath growth. Typical leaf symptoms are irregular to round eyespots, 1-3 mm in diameter, with light-brown to bleached centers and darker brown margins, often with a surrounding chlorotic area. The disease also occurs in the form of a pepper spot resembling that produced on *T. repens* by *L. trifolii*. Petioles as well as the leaf blades may be infected. In the Northeast, *Leptosphaerulina* leaf spot is severe during the summers in cool, wet seasons; in the South, it occurs only during the spring, and the pepper spot phase of the disease is most common. The annual *Medicago* spp., particularly *M. hispida*, also are attacked.

In the field, *L. trifolii* generally is restricted to *Trifolium* spp. and *L. briosiana* to *Medicago* spp. with only traces of cross-infection. In Pennsylvania during the spring, when *T. repens* is most severely infected with pepper spot, *M. sativa* is practically free of *Leptosphaerulina* leaf spot. During June and July when *Leptosphaerulina* leaf spot causes severe defoliation of *M. sativa*, only traces of pepper spot can be found on *T. repens* in neighboring fields. In Georgia during the spring when both diseases occur and produce similar pepper spot symptoms, *L. trifolii* is consistently isolated from *T. repens* and *L. briosiana* from adjacent plants of *M. sativa*. Similarly, *L. trifolii* has been present on *T. repens* in Great Britain for many years (41); whereas *L. briosiana* was first reported on *M. sativa* in 1956. The source of infection in this case was traced to imported seed (6).

Leaf spot of peanut (*Arachis hypogaea* L.) caused by *L. arachidicola* occurs commonly in Georgia and probably in other southeastern states throughout the summer (20). Symptoms usually are pepper spots, although the dark-brown lesions may attain diameters of 3 mm. More severe symptoms of leaf scorch on *A. hypogaea* have been reported in Taiwan (49) and have been produced by artificial inoculation in Georgia (20).

Three additional *Leptosphaerulina* spp. (*L. australis*, *L. americana*, and *L. argentinensis*) have been found on dead tissue of forage legumes, grasses, and other plants; but these species seemed secondary saprobes. In this study, severe damage in the form of large, tan leaf blotches was encountered in a roadside planting of Kudzu (*Pueraria lobata*) in Georgia. Ascocarps of *L. argentinensis* were consistently present in the dead tissue. A similar necrosis, however, occurred on leaves of various other plants in the area; except on *Sorghum halepense*, *L. argentinensis* was absent from the dead tissue. The damage apparently resulted from the application of a weed killer. Tehon and Stout (45) attributed a leaf spot of *Vigna sinensis* (Torner) Savi in Illinois to *L. australis* (*L. vignae*) but offered no proof of the pathogenicity of the fungus.

PHYSIOLOGY.—When infected host leaves were placed on moist filter paper in petri dishes, ascocarps of *Leptosphaerulina* spp. usually developed within 5 days and discharged ascospores upward on the lid of the petri dish. Cultures were easily obtained by discharging the spores on agar plates. Isolations of pathogenic species were made by planting bits of infected tissue surface-sterilized in 0.5% sodium hypochlorite on plates of water agar or 20% V-8-juice agar. Cultures were maintained on slants of V-8-juice agar or in tubes of autoclaved soil. The latter proved useful in preparing inoculum. When grains of soil from the tube cultures were scattered over an agar plate, the surface was covered with sporulating colonies in a short time.

In preliminary tests, isolates of *L. briosiana* and *L. trifolii* were grown on plates of 6 agar media and incubated in the laboratory. Of these media, 20% V-8 juice agar (22, 25) proved the most satisfactory for sporulation and was used in all subsequent cultural studies. Numerous ascospores were produced when the room temperature remained below 25°C, whereas sporulation was erratic at higher temperatures. Apparently, light also affected sporulation, since cultures receiving little light produced few ascospores. To demonstrate the effect of various environments on growth and sporulation and to determine differences in cultural characteristics among the 6 *Leptosphaerulina* spp., further experiments were conducted under controlled conditions.

*Effect of temperature on growth rate.*—To determine the optimum temperature for mycelial growth, petri-dish cultures were placed in incubators at temperatures of 5-35°C, at 5° intervals. Included in these tests were 9 isolates of *L. trifolii*, 7 of *L. briosiana*, 1 of *L. argentinensis*, and 2 each of *L. arachidicola*, *L. australis*, and *L. americana*. Maximum radial growth of *L. briosiana* and *L. trifolii* occurred at 20-25°C. At 15°C, growth of *L. trifolii* was relatively greater than that of *L. briosiana*. No differences in response to temperature were evident between isolates of these 2 species from the Northeast and the Southeast. Isolates of *L. americana* and *L. argentinensis* likewise produced maximum growth at 20-25°C. Isolates of *L. australis* grew well from 15-25°C with a maximum at 20°C,

whereas maximum growth of *L. arachidicola* occurred at 25-30°C.

In a comparison of growth rates of the 6 *Leptosphaerulina* spp., isolates were grown at a constant temperature of 20°C under continuous fluorescent light for 5 days. Discs of agar, 3 mm in diameter, were taken from the periphery of actively growing colonies to inoculate the plates. Colony diameters were recorded at the end of the incubation period (Table 2). Because of variation among isolates within a species, only *L. trifolii* differed significantly in average growth rate. Isolates of *L. briosiana* also were slow-growing, but the average was nearly double that for *L. trifolii*. The 3 saprobic species (*L. australis*, *L. argentinensis*, and *L. americana*) grew more rapidly in culture than the other 3.

*Effect of temperature on ascospore production and germination.*—In determining the optimum temperature for ascospore formation, the same isolates used in the growth rate studies were grown under continuous fluorescent light at 15°, 20°, 25°, and 30°C. After 5 days, mounts were made from each culture to determine the stage of development of ascospores.

At 15°C, most cultures of *L. briosiana* and *L. trifolii* reached only the stage of ascus formation in the 5-day period, whereas at 20°C both species produced mature ascospores. Ascospores of *L. briosiana* were farther developed than those of *L. trifolii*, a possible reflection of the slower growth rate of the latter. Some isolates of both species progressed as well at 25°C, but this temperature was more favorable for *L. briosiana* than for *L. trifolii*. Ascocarps developed at 30°C, but no asci or ascospores were produced. A greater number of ascocarps of *L. briosiana* developed at 20° and 25°C, whereas more ascocarps of *L. trifolii* formed at 15° and 20°C. *L. arachidicola* produced numerous ascospores at 25° and 30°C, but almost none at 15° and 20°C. *L. australis* produced immature ascospores at 15°C, numerous mature ascospores at 20° and 25°C, and a few immature ascospores at 30°C. Mature ascospores formed in cultures of *L. americana* and *L. argentinensis* at 20° and 25°C.

In an attempt to distinguish between the effects of temperature on ascospore formation and on ascospore ejaculation, cultures of each isolate grown at 15°, 20°, and 25°C were placed in a refrigerator at 5°C for 1 day. Two cultures of each isolate were divided, and half of each colony was transferred to another petri dish, The half colonies were incubated at 15°, 20°, 25°, and 30°C. Following a method devised by L. W. Baxter (personal correspondence), after 12 and 18 hours each culture was inverted over a petri dish of clear agar for 1 minute to permit ascospore discharge. These plates were incubated at room temperature for 1-2 days and the individual colonies were counted as an indication of the number of ascospores discharged.

All isolates of *L. trifolii* discharged most ascospores at 20°C. A moderate number were discharged at 15°, fewer at 25°, and least at 30°C. Ascospore discharge by isolates of *L. briosiana* was less affected by temperature, being high at 15°, 20°, and 25° and moderate even at 30°C. Ascospore discharge by *L. arachidicola* was most abundant at 25° and 30°C. Isolates of *L. australis* discharged ascospores abundantly at all temperatures, with most at 20° and 25°C. Ascospore discharge by isolates of *L. americana* and *L. argentinensis* was not great but occurred at all temperatures, the maximum at 20°C.

The optimum temperatures for ascospore germination of only 2 species (*L. trifolii* and *L. briosiana*) were determined by incubating spore suspensions on glass slides in petri-dish moist chambers and also by naturally discharged ascospores on agar at temperatures of 15-30°C for 3-4 hours. In terms of percentage germination and germ-tube length, the optimum for both species was between 20° and 25°C. Again, the optimum for *L. briosiana* was slightly higher than that for *L. trifolii*.

*Effect of light on sporulation.*—Petri-dish cultures of isolates of all 6 *Leptosphaerulina* spp. were grown for 16 days at a constant temperature of 20°C in total darkness and under continuous fluorescent light. The majority of the isolates of *L. briosiana*, *L. trifolii*, and *L. arachidicola* produced ascospores only when exposed to light. Of the 11 isolates of *L. briosiana*, only 2, both of which were prolific sporulators, produced ascospores in total darkness. A single isolate of *L. trifolii* produced a very few ascospores in the absence of light. The ascospores developed only in a sector of 1 colony of this isolate. A single isolate of *L. arachidicola* from Taiwan sporulated well in darkness, whereas the isolates from Georgia sporulated only in light. The remaining species (*L. australis*, *L. americana*, and *L. argentinensis*) produced ascospores in both darkness and light.

*Colony characteristics.*—In addition to the strikingly slower growth rate, colonies of *L. trifolii* were distinct in appearance from those of other species. The surface of the colonies was covered with an almost solid crust of large black ascocarps, and the colonies showed no zonation when grown in alternating light and darkness. All other species were essentially similar in culture, producing spreading colonies of dark appressed mycelium on which the generally smaller ascocarps, although numerous, were more dispersed than in colonies of *L. trifolii*. When exposed to alternating light and darkness, the colonies exhibited zonation, since relatively fewer ascocarps, even of isolates capable of sporulating in the absence of light, developed in the dark. There were variations among these species, such as the relatively smaller colony diameter of most isolates of *L. briosiana* and the tendency of isolates of *L. americana* to produce fewer, scattered ascocarps and a greater amount of aerial mycelium. These differences, however, were not consistent enough to furnish dependable criteria for recognizing species. Another striking character was the production in the agar of a pink pigment by isolates of *L. australis*. Pigmentation could be demonstrated most easily by comparing cultures grown on slants in culture tubes. The original reddish color of the V-8-juice agar rapid-

TABLE 3. Incidence of disease on legume seedlings inoculated with isolates of 3 *Leptosphaerulina* spp.

| Legume seedling | Fungus spp. and host source | | | | | | |
|---|---|---|---|---|---|---|---|
| | *L. trifolii* | | | *L. briosiana* | | | *L. arachidicola* |
| | *Trifolium repens* | *T. pratense* | *T. hydridum* | *Medicago sativa* | *M. lupulina* | *M. hispida* | *Arachis hypogaea* |
| *M. sativa* | 4 S-M[a] | 3 S-M | 3 S | 5 L | 4 M-L | 6 M | 0 |
| *M. lupulina* | 2 S | 3 S-M | | 4 L | 3 M-L | 3 M | |
| *T. repens* | 7 L | 5 M-L | 3 M | 2 S | 1 S | 3 S | 0 |
| *T. pratense* | 6 L | 6 L | 2 L | 3 S | | | |
| *Melilotus alba* | 5 L | 6 L | | 1 S | | | |
| *Pisum sativum* | 6 L | | | 2 S | | | |
| *Vigna sinensis* | 4 M | | | tr | | | |
| *Lathyrus latifolius* | 4 M | | | 2 S | | | |
| *Coronilla varia* | 1 S | | | tr | | | |
| *A. hypogaea* | 0 | | | 0 | 0 | 0 | 7 L |

[a] Disease index based on a scale of 0-7: O=no disease and 7= numerous lesions; L= large leaf lesions; M= medium-size lesions; S= small lesions.

ly bleached out in light and was replaced by a faint pink color beneath colonies of *L. australis*. The pigment was not produced in cultures on potato-dextrose agar.

PATHOGENICITY.—The failure of early workers (16, 23, 24) to obtain typical symptoms of leaf spot of *Medicago sativa* following inoculations with *L. briosiana* led to conflicting opinions on the pathogenicity of the fungus (16, 24). Success obtained by suspending infected host leaves on which the fungus was fruiting over seedlings (15, 23) led to inoculations with ascospore showers from petri-dish cultures of the fungus inverted over the plants (1, 22).

In the early stages of the present study, results of inoculations were erratic because of insufficient inoculum, method of application, and the use of older host plants, which are more resistant than seedlings. The following method was developed for routine use in inoculation studies. Host seedlings were grown in 4-in. pots and inoculated when 8 weeks old. The soil was moistened, and the seedlings were sprayed with a fine mist of water. A chicken-wire cylinder, 6 in. high, was fitted inside the pot to support an inverted petri dish containing vigorously sporulating colonies of the fungus over the seedlings. The seedlings, culture, and wire support were covered with a polyethylene bag sealed around the pot with a rubber band. Greenhouse temperatures were maintained at 16-24°C. While covered, the plants were protected from direct sunlight. Cultures were removed after 8-24 hours and bags after 24-48 hours. Tiny lesions often were evident within 24 hours.

*Cross-inoculations.*—Numerous inoculations were made on host seedlings with 1-6 isolates of the 6 *Leptosphaerulina* spp. from various sources. Results are shown in Table 3. *L. australis*, *L. americana*, and *L. argentinensis* were omitted from the table because isolates of these 3 species failed to produce infection on any of the seedlings tested. *L. arachidicola* caused severe infection only on *Arachis hypogaea*. *L. trifolii*, and to a lesser extent *L. briosiana*, showed wide host ranges producing at least a trace of infection on seedlings of all legumes except *A. hypogaea*. Isolates of each species from different hosts varied no more in pathogenicity than did different isolates from the same host, apparently in both cases because of differences in sporulation. Cross-infection occurred with *L. briosiana* and *L. trifolii* on *M. sativa* and *T. repens*, but symptoms appeared earlier and disease was more severe on the original host. Lesions caused by *L. briosiana* on *M. sativa* enlarged and chlorotic areas developed around each lesion. Although *L. trifolii* was moderately pathogenic to *M. sativa*, the lesions usually remained small and no chlorosis developed. *L. trifolii* also caused severe infection on *Melilotus alba* and *Pisum sativum* and moderate infection on *Vigna sinensis* and *Lathyrus latifolius* L., plants on which *L. briosiana* produced little or no disease. None of the isolates attacked seedlings of *Bromus inermis* Leyss or *Lycopersicon esculentum* Mill.

Because *L. argentinensis* was found on spotted leaves of *Pueraria lobata* in the field, further inoculations were made to determine its pathogenicity to this species. Isolates from *P. lobata* and *Melilotus alba*, as well as isolates of *L. arachidicola*, *L. australis*, and *L. americana*, failed to produce any infection on *P. lobata*. Isolates of *L. trifolii* and *L. briosiana*, however, caused a moderately severe pepper spot on the young leaves of this species.

Other series of inoculations were made on detached host leaves. The leaves were floated on water in petri dishes held in a growth chamber under fluorescent light at approximately 20°C. Sporulating cultures were inverted over the leaves for 24 hours and were then replaced with the petri-dish lids. Lesions in the form of pepper spots appeared within 24 hours. If infections were severe, areas of the leaf died almost immediately and mature ascocarps developed in 5 days. Isolates of *L. trifolii* caused death of the leaf tissue accompanied by fruiting of the fungus on *Trifolium repens*, *T. incarnatum*, *Medicago sativa*, *Pisum sativum*, *P. sativum* L. var. *arvense* (L.) Poir., and *Phaseolus vulgaris* L. and pepper spot on *M. hispida* and *Glycine max* (L.) Merr.; but they failed to infect

*Arachis hypogaea*, *Lespedeza* sp., and *Rosa* sp. Most isolates of *L. briosiana* produced necrosis only on *M. sativa* and caused pepper spot on all other hosts infected by *L. trifolii*. A single isolate also produced necrosis on *M. hispida*, *P. sativum*, *P. sativum* var. *arvense*, and *Phaseolus vulgaris*. *L. arachidicola* caused lesions only on *A. hypogaea*, except for a possible trace of infection on *P. vulgaris*. *L. australis* and *L. argentinensis* produced no symptoms on any species. *L. australis*, which occurs frequently on dead grass leaves, failed to infect living segments of *Digitaria* leaves. When the leaves were killed in boiling water before they were inoculated, *L. australis* fruited in 5 days as it did on boiled leaves of *A. hypogaea* and *T. repens*. *L. trifolii* and *L. briosiana* also fruited rapidly on boiled leaves of *A. hypogaea* and *Digitaria* although they did not infect living leaves. None of these fungi, however, fruited on boiled *Rosa* leaves.

*Effect of temperature on incidence of disease.*—A series of tests was conducted to determine the optimum temperature for infection and disease development of 2 isolates of *L. trifolii* on seedlings of *T. repens*. Plants were kept in a sectional meat case at 15-30°C for inoculation and subsequent disease development. Most infection appeared on plants at 15-22°C.

In other tests with single isolates of *L. trifolii* and *L. briosiana* on *T. repens* and *M. sativa*, respectively, 3 pots of seedlings of each host were placed in incubators at 15°, 20°, and 25°C for 5 hours. The plants then were grouped under sporulating cultures of each fungus. After 3 hours, all plants were sprayed lightly with water, covered with polyethylene sacks, and returned to the proper incubators. Fluorescent lights were turned on after 12 hours. Within 24 hours, numerous small lesions were evident on the *T. repens* leaves at all temperatures. After 7 days, the lesions had enlarged to diameters of 2-3 mm at 15° and 20° and to a smaller size at 25°C. Lesions developed on *M. sativa* leaves within 24 hours at 20° and 25°C. After 7 days, the lesions were 2-4 mm in diameter at the higher temperatures and slightly less at 15°C.

DISCUSSION.—The genus *Leptosphaerulina* comprises 2 ecological groups of species. One group, including *L. trifolii*, *L. briosiana*, and *L. arachidicola*, consists of pathogens capable of initiating infections but fruiting only in the necrotic tissue. A second group, composed of *L. australis*, *L. americana*, and *L. argentinensis*, is made up of species which, although saprobic, are closely related ecologically to the pathogens, since they quickly invade and fruit on necrotic tissue and often give the appearance of being responsible for the necrosis. The pathogenicity of *Leptosphaerulina* spp. may be readily demonstrated by inoculations with naturally discharged ascospores. Similar ascospore showers probably occur in nature as evidenced by the multiplicity of tiny lesions, each originating from a single ascospore, that occur on host leaves. The severity of disease depends primarily on the supply of inoculum in the form of air-borne ascospores. Although the possible effect of environment on the host and on the host-pathogen interaction should not be ignored, the seasonal occurrence of *Leptosphaerulina* leaf spots appears related to the effects of temperature on sporulation of the pathogens. Although the diseases caused by *L. trifolii* and *L. briosiana* develop only during cool weather, the restriction of *L. trifolii* to the coolest periods of the growing season is reflected in the slightly lower temperature requirements of this species. Only *L. arachidicola*, which is capable of sporulating at 30°C, causes leaf spot in warm weather; and the severity of this disease is probably limited by the high temperatures under which the host is commonly grown.

The host ranges of the pathogenic species in nature (except *L. arachidicola* which apparently is pathogenic only to *Arachis* spp.) also appear influenced by their temperature requirements. Although *L. briosiana* commonly occurs only on *Medicago* spp. in the field, inoculations demonstrate that it is capable of causing a mild infection on many other legumes. It does, in fact, occasionally occur on *Trifolium* spp., but its host range under more competitive conditions in the field is narrower than might be expected from the results of experimental inoculations. *L. trifolii* is even more limited in the field since it has been reported only on *Trifolium* spp. Inoculation experiments, however, indicate that it has the greatest potential host range of all species tested, causing severe to moderate infection on plants such as *Melilotus alba*, *Pisum sativum*, *Vigna sinensis*, *Lathyrus latifolius*, and *Pueraria lobata*. Plants of species such as *M. alba*, *V. sinensis*, and *P. lobata* may escape infection because their major period of growth occurs during the summer when high temperatures are unfavorable for sporulation of *L. trifolii*. The cultural conditions under which annual crops are grown also may be less favorable, for survival of the fungus and for development of a sufficient population to produce the enormous numbers of ascospores required for conspicuous development of the disease, than those afforded by a perennial species such as *T. repens*.

The occurrence in *Leptosphaerulina* of saprobes and of pathogens differing in host relationships, symptoms produced, and seasonal occurrence demands a consideration of species classification. Petrak (32) reduced *L. briosiana* to synonomy with *L. trifolii*, and Allison, *et al.* (1) included both of these species under *L. americana* (*Pleosphaerulina hyalospora*). Because of the difficulties encountered in species delimitation, lumping is a tempting solution to the problem. It is hardly practical, however, to classify the saprobic *L. americana* with large, 6-septate ascospores and the pathogenic *L. briosiana* with small, 4-septate ascospores as a single species. The primary separation of species on the basis of morphological characters proved difficult because of the extreme variability, even in ascospore characteristics, within species and within single isolates. *L. australis* and *L. americana*, representing extremes in ascospore size and septation, are distinct, but both overlap with the intermediate *L. argentinensis*.

Reliance must be placed on a combination of tendencies in ascospore size and septation rather than on a single clear-cut character. Some support for the species separations arrived at derives from differences in cultural characteristics, temperature requirements, and pathogenicity. *L. australis* and *L. arachidicola*, which exhibit only subtle differences in morphology, differ in pigment production in culture and in pathogenicity. *L. arachidicola* offers the clearest example of host specificity among the species studied. *L. briosiana* and *L. trifolii* are easily distinguished by colony characteristics in culture; *L. trifolii* differs from all other species in growth rate and appearance. The differences in pathogenicity, as demonstrated by artificial inoculations, are not as sharp as might be expected from field observations. As indicated by Allison, *et al.* (1) cross-infection with these species occurs on *Trifolium* and *Medicago* spp. *L. trifolii* and *L. briosiana*, however, differ in severity of infection and symptoms they produce on hosts of these 2 genera. They also differ decidedly in pathogenicity on other legumes, such as *Melilotus alba* and *Pisum sativum*.

Other species have been described in *Leptosphaerulina* and many additional species which should be included in *Leptosphaerulina* undoubtedly have been placed in various other genera, such as *Pleosphaerulina* and *Pleospora*. *L. pulchra* is an example of 1 such species which is readily distinguished by the unusual shape of its asymmetrical ascospores (2). Difficulties may be anticipated, however, in extending the classification developed here to other species, especially to those represented only by herbarium specimens.

A key to LEPTOSPHAERULINA spp.:

1. Ascospores 3-5-septate ..... 2
1. Ascospores 5-8-septate ..... 5

2. Ascospores most frequently 3-septate, tending to be phragmosporous with means of 0-1 longitudinal septa, large, maximum length up to 60 $\mu$, maximum diameter more than 20 $\mu$, mean diameter more than 17 $\mu$; colonies slow-growing (less than 3 mm radial growth/day), covered with a black crust of large, often compound ascocarps; pathogenic primarily on *Trifolium* spp., but capable of infecting other legumes ..... *L. trifolii*

2. Ascospores most frequently 4-septate, usually muriform with means of 1-2 longitudinal septa, medium-sized to small, maximum length up to 50 $\mu$, maximum diameter less than 20 $\mu$, mean diameter less than 17 $\mu$; colonies fast-growing (more than 4 mm radial growth/day), covered with small, dispersed ascocarps ..... 3

3. Ascospores medium-sized, maximum length up to 50 $\mu$, maximum diameter more than 17 $\mu$, mean diameter more than 16 $\mu$; pathogenic primarily on *Medicago* spp. but capable of infecting other legumes ..... *L. briosiana*

3. Ascospores small, maximum length up to 40 $\mu$, maximum diameter 17 $\mu$ or less, mean diameter 14 $\mu$ or less ..... 4

4. Ascospores consistently muriform; producing a pink pigment on V-8-juice agar; saprobic on dead leaves of legumes, grasses, and other plants ..... *L. australis*

4. Ascospores dimorphic, producing both muriform, ellipsoid ascospores and a smaller percentage of phragmosporous, cylindrical ascospores; not producing a pink pigment in culture, pathogenic on *Arachis* ..... *L. arachidicola*

5. Ascospores most frequently 6-septate and muriform with 4-5 longitudinal septa; saprobic on dead leaves of *Vicia, Pisum, Trifolium*, and *Phleum* ..... *L. americana*

5. Ascospores most frequently 5-septate and muriform with 3 longitudinal septa; saprobic on dead leaves of *Melilotus, Pueraria, Sorghum*, and *Datura* ..... *L. argentinensis*

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# SAPROPHYTIC BEHAVIOR OF RHIZOCTONIA IN SOIL

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SUMMARY

Saprophytic activity of *Rhizoctonia*, indicated by percentage of buckwheat stem pieces colonized in unsterilized *Rhizoctonia*-infested soils, was significantly higher when soil moisture was maintained at 20-50% of the moisture-holding capacity than when maintained at 60-90%. In a soil from Maryland, the saprophytic activity was greatest when the soil was incubated at 20°C, whereas in a Florida soil it was greatest when the soil was incubated at 26-30°C. The length of incubation of the substrate strongly influenced saprophytic activity. Buckwheat and oat stem pieces incubated in *Rhizoctonia*-infested soils for 2-4 days had the highest percentage of colonization. Saprophytic survival decreased with prolonged incubation, but the fungus was still recoverable from the colonized substrates after 120 days in soil. Soil reaction and the kind of inoculum and its length of incubation in unsterilized soil also affected saprophytic activity and survival of *Rhizoctonia*. Substrate enrichment with nitrogen (carbon/nitrogen ratios of about 5-50) increased saprophytic activity and survival of *Rhizoctonia*, whereas substrate enrichment with carbon decreased them. Supplemental phosphorus and calcium had no apparent effect on the saprophytic activity. *R. solani* assumed a parasitic status from saprophytically colonized buckwheat stem pieces in both sterilized and unsterilized soil.

During the last 10 years, considerable information has been accumulated on the saprophytic life of some soil-borne organisms associated with root-rot diseases of plants. The Cambridge Botany School has reported extensively on the saprophytic behavior of some cereal root-rot fungi (5, 6, 7, 13). Studies on the saprophytic life of the majority of root-rot fungi, however, have been limited in number and scope.

*Rhizoctonia*, a soil-inhabiting fungus, can generally carry on an active saprophytic existence in the soil and survive in the absence of its host or hosts by competitive colonization of dead soil organic matter (4, 11).

Very little, however, is known of its behavior as a soil saprophyte, because most of the studies on the saprophytic growth of *Rhizoctonia* have been carried out in pure cultures or in sterilized soils.

Blair (3) studied the behavior of *R. solani* Kühn in natural unsterilized soils by the Rossi-Cholodny soil-plate method. He demonstrated the ability of this pathogen to make free and independent growth through unsterilized soil. Soil moisture, aeration, and texture, and the influence of organic supplements upon growth of *Rhizoctonia* received much attention in his experiments.

In spite of Blair's (3) findings, very little is known of the ability of *Rhizoctonia* to colonize various organic substrates in soil and of the factors affecting the saprophytic activity and survival of this pathogen in natural, unsterilized soils. The Rossi-Cholodny slide, though useful in many respects, is an unsatisfactory substitute for organic matter surfaces available for colonization in soil.

Adequate control measures for diseases caused by soil-inhabiting fungi, such as *Rhizoctonia*, cannot be devised unless the ecology of both the parasitic and the saprophytic phases of such primitive parasites has been studied adequately. Thus, it seemed advisable to investigate the saprophytic behavior of *Rhizoctonia* in natural, unsterilized soils. Results of such investigations are herein reported. The effect of a single soil ecological factor, $CO_2$, on the saprophytic behavior of this pathogen will be discussed in a separate report. A preliminary report of the present investigation was published (16).

Materials and Methods.—The soils used were a greenhouse loamy sand (GLS), pH 6.2; Elsinboro sandy loam (ESL), pH 5.8; and Immokalee fine sand (IFS), pH 5.6. GLS had 0.10% total N and a carbon/nitrogen (C/N) ratio of 20.4. ELS had 0.11% total N and a C/N ratio of 22.2. All 3 soils were naturally infested with *Rhizoctonia*. Continuous cropping with susceptible plants (beans, sugar beets, *etc.*) so increased the inoculum potential in these soils that more than 50% of buried buckwheat stem pieces were colonized.

In some experiments, soils were artificially infested with known isolates of *R. solani* and *R. praticola* (*Pellicularia praticola* (Kotila) Flentje). The isolates were grown separately in 300-ml, wide-mouth jars on a sterilized mixture of vermiculite and maize-meal (90% vermiculite and 10% maize-meal by weight, water to 20% moisture, w/v). After incubation for 28 days, the replicates of each isolate were mixed and added to soil at the rate of 1% (w/w).

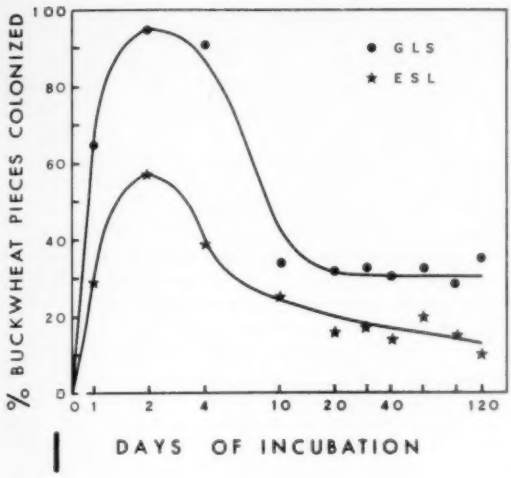
Naturally infested soils were partially air-dried shortly before use, passed through a 5-mm sieve, mixed thoroughly, weighed into portions, and placed in No. 3 tin cans or 300-ml, wide-mouth jars. Artificially inoculated soils were dried and sifted prior to inoculation. The equivalent of 800 and 300 g of oven-dry soil was placed in the cans and jars, respectively. Unless otherwise indicated, the soils were adjusted to approximately 50% of their moisture-holding capacity (MHC) and incubated at 22-24°C. Soil moisture determinations were made at the beginning and end of each test. In experiments of short duration (2-4 days), no further adjustment of the soil moisture content was necessary. In experiments lasting more than 4 days, soils were maintained at 50% MHC throughout by daily weighing and watering.

The competitive saprophytic activity of *Rhizoctonia* was determined by incubating, for certain time intervals, mature buckwheat or oat stem pieces (5-8 mm long) at the rate of 4 g of pieces/1 kg of naturally infested or artificially inoculated soil. The saprophytic activity was expressed as percentage of pieces colonized by species of *Rhizoctonia*, assessed by culturing 50 pieces/replication on water agar plates (15). In these studies, competitive saprophytic activity is a percentage expression of Garrett's (11) competitive saprophytic ability. The buckwheat and oats used as baits were grown at Beltsville and harvested at an advanced stage of maturity. Buckwheat stem pieces contained 34.5% total C and 0.36% total N. The oat stem pieces contained 36.2% total C and 0.44% total N. The C/N ratios of buckwheat and oat stem pieces were 95 and 83, respectively. Total N and C of both soil and plant tissue were determined by a micro-Kjeldahl method and by dry combustion, respectively.

The effect of soil reaction on the saprophytic activity of *Rhizoctonia* was studied by dividing GLS and ESL into 3-kg portions and adjusting the soils with $Ca(OH)_2$ or $Al_2(SO_4)_3 \cdot 18H_2O$ to give a reaction range of pH 3.5–9.0. In preliminary experiments, neither the Ca nor the $SO_4$ ions *per se* affected the saprophytic activity of the pathogen. The chemicals were mixed thoroughly with the soil portions and enough water was added to bring the soil moisture to 40% MHC. After 3 days of incubation, inoculum of *R. solani* was mixed with the soil. After 3 additional days of incubation, the soil portions of each pH level were divided into four 700-g replicates, placed in No. 3 tin cans, and mixed with 3 g of buckwheat stem pieces. Enough water was added to raise the soil moisture to 50% MHC.

In some experiments, buckwheat stem pieces were used without changing their nutrient value. In other experiments, the C/N ratios of the pieces were modified by steeping them for 24 hours in glucose or $NaNO_3$ solutions calculated to produce a C/N ratio range of 5–120. In 1 experiment, supplemental C and N were added by soaking the pieces in glucose and $NaNO_3$ solutions to give an additional 1 or 3% each of C and N, respectively, on the oven-dry weight basis of the substrate. *Rhizoctonia* spp. were not present among the microorganisms found on the substrates before their use in soil.

The ability of *R. solani* to assume a parasitic status from a saprophytically colonized substrate was tested by determining its ability to cause damping-off and infection of radish seedlings. "Buckwheat inoculum" was prepared by mixing autoclaved soil, buckwheat



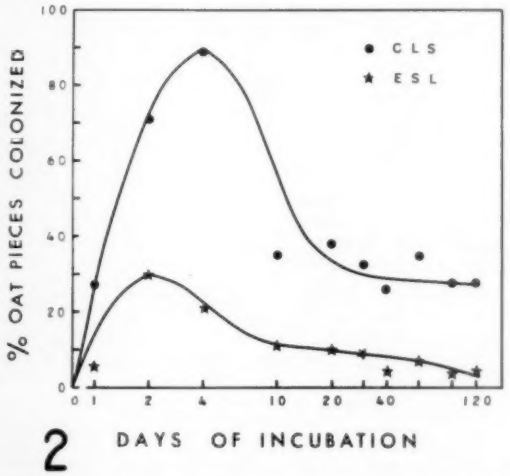




**Fig. 1-2.** Competitive saprophytic colonization by *Rhizoctonia* of mature buckwheat and oat stem pieces buried for various lengths of time in 2 soils naturally infested with the pathogen. **1)** Buckwheat stem pieces. **2)** Oat stem pieces.

stem pieces, and maize-meal vermiculite inoculum (100 g soil at 50% MHC, 1 g buckwheat stem pieces, 1 g inoculum) of isolate 7, pathogenic to radish. After 4 days of incubation, the pieces were harvested on a 5-mm screen, washed for 10 minutes with running tap water, and blotted dry. With this method, 95–100% of the pieces were internally colonized by the pathogen. Autoclaved and nonautoclaved, *Rhizoctonia*-free GLS was inoculated with "buckwheat inoculum" at 2 concentrations. Low inoculum consisted of 3 colonized buckwheat stem pieces buried 1 cm below the soil surface and near the center of coffee cans containing the equivalent of 1 kg of oven-dry soil. High inoculum consisted of 30 colonized pieces mixed with the equivalent of 1 kg of oven-dry soil. One week after the inoculum was mixed with the soil, surface-disinfected radish seeds were planted at the rate of 25 seeds/coffee can. Seedling emergence was determined 5 and 10 days after planting. Infection of emerged seedlings was indexed 21 days after planting by rating each individual plant on an infection scale from 0 (no visible infection) to 5 (plants completely girdled). Immediately after the seedlings were removed and rated, the soil was air-dried and passed through a 5-mm screen to remove root fragments and the "buckwheat inoculum." Fresh buckwheat stem pieces were mixed with the soil to determine the saprophytic activity.

RESULTS.—*Effect of length of substrate incubation in soil on the saprophytic activity.*—Buckwheat and oat stem pieces were incubated for certain time intervals in GLS and ESL, both naturally infested with *Rhizoctonia*. The peak of saprophytic activity on buckwheat stem pieces occurred after 2 days of incubation of the pieces in both soils (Fig. 1). The peak of saprophytic activity on oat stem pieces occurred after 2 days of incubation in ESL and after 4 days in GLS (Fig. 2). *Rhizoctonia* in GLS had a higher saprophytic activity than in ESL. Buckwheat stem pieces were more suitable for colonization by *Rhizoctonia* in both soils than oat stem pieces. Both substrates incubated for 120 days in both soils contained viable *Rhizoctonia*, despite the advanced stage of tissue decomposition. After the brief initial and intensive saprophytic activity of the pathogen, a period of declining activity was observed beginning at about the 3-4th day of incubation (Fig. 1, 2). Between the 10th and 120th day of incubation, there was a slow decline of the pathogen in ESL, but practically no decline in GLS. This experiment was done twice. In both instances, it was discontinued after 120 days of incubation, because the pieces had deteriorated to such an extent that handling became impossible.

Microscopic examinations of hand-made tissue sections revealed the presence of *Rhizoctonia* mycelium in the pieces that were incubated for at least 2 or 3 days in either soil. The establishment of mycelium in the tissue and the kind of curves shown in Fig. 1, 2 indicate that there was actual colonization of the substrates and not a simple trapping in the tissues of soil particles carrying the fungus hyphae.

*Effect of soil moisture.*—The competitive saprophytic activity of *Rhizoctonia* in naturally infested GLS and ESL was significantly higher when the soil moisture was maintained at 20, 30, 40, or 50% MHC than when it was maintained at moisture contents higher than 50% (Table 1). At 70 and 80% MHC, there was an appreciable reduction of colonization; at 90%, saprophytic activity was almost eliminated. At the end of the 4-day test period, soil moisture was uniformly distributed in all 4 replications of each treatment except at 20% MHC, the lowest moisture tested. At this moisture level, there was some accumulation of moisture around the buckwheat stem pieces due to their

TABLE 1. Effect of soil moisture level on competitive saprophytic colonization by *Rhizoctonia* of mature buckwheat stem pieces buried for 4 days in 2 soils naturally infested with the pathogen

| % of soil MHC | Saprophytic colonization[a] in stated soil | |
|---|---|---|
| | Greenhouse loamy sand | Elsinboro sandy loam |
| 20 | 66 a[b] | 82 a |
| 30 | 67 a | 86 a |
| 40 | 72 a | 85 a |
| 50 | 69 a | 81 a |
| 60 | 67 a | 70 b |
| 70 | 44 b | 49 c |
| 80 | 23 c | 34 d |
| 90 | 9 d | 3 e |

[a]Percentage of buried mature buckwheat stem pieces colonized.

[b]Numbers followed by the same letter are not significantly different at the 5% level of probability.

natural hygroscopicity. Thus, the actual moisture status near the pieces may have exceeded 20% MHC.

*Effect of soil temperature.*—Buckwheat stem pieces were buried in 2 soils and incubated for 4 days at various temperatures ranging in 2° increments from 18–34°C. Significantly greater activity of *Rhizoctonia* in GLS occurred at 20°C than at all other temperatures tested. In this soil, activity decreased markedly at 30°C. In IFS, however, a Florida soil naturally infested with the pathogen, *Rhizoctonia* was most active saprophytically at 26–30°C and significantly less active above and below these temperatures.

*Effect of soil reaction.*—Although the effect of soil reaction on growth and disease-inducing ability of *R. solani* was extensively studied in the past, only Blair's (3) work dealt directly with the effect of soil pH on the growth of 2 isolates of the pathogen on Rossi-Cholodny slides buried in unsterilized soils. In the present work, emphasis was placed on soil reaction influencing the ability of a number of isolates of *R. solani* to colonize a substrate in unsterilized soils saprophytically.

In both GLS and ESL, good saprophytic growth of the pathogen was made at a soil reaction range of pH 4.5–8.1. In both soils, colonization declined to less than 10% at pH values of 4.1 in GLS and 3.7 in ESL. Optimum growth in both soils was observed at a neutral or slightly alkaline reaction. These results confirm those of Blair (3).

*Effect of length of inoculum incubation in soil.*—Five isolates of *Rhizoctonia* were used: isolate R 1, from hypocotyl of bean seedling at Beltsville; isolate R 5 (subsequently identified by the authors as *R. praticola*) from damped-off sugar beet seedling obtained through the courtesy of C. L. Schneider, Beltsville; isolate R 6 from sweet clover, and R 7 from soybeans, both through the courtesy of L. J. Herr, Ohio Agricultural Experiment Station; and isolate R 8, very pathogenic to radish, from buckwheat stem piece colonized in infested GLS.

The saprophytic activity of the 5 isolates decreased with length of incubation in the soil, but the rate of decrease varied among the isolates involved (Table 2). Two weeks of incubation in ESL maintained at 50% MHC did not change the saprophytic activity of the 5 isolates tested. After 28 weeks of incubation, however, the loss in activity ranged from 24% (R 7)–91% (R 1).

*Effect of supplemental nutrients.*—Supplemental P from $Na_2HPO_4 \cdot H_2O$ up to 0.5% of the oven-dry weight of the substrates (buckwheat and oat straw pieces), or supplemental Ca from $CaCl_2$ up to 2,000 ppm additional Ca in the soil, had no apparent effect on the saprophytic colonization by *Rhizoctonia* in 3 soils naturally infested with the pathogen. Addition of N from $NaNO_3$ to buckwheat or oat stem pieces, however, produced a marked increase in the saprophytic colonization by *Rhizoctonia* in unsterilized, naturally infested GLS (Table 3). The percentage of colonization of N-enriched pieces by the pathogen was not reduced by the addition of 1 or 3% C from glucose. Supplemental C, however, added alone to either oat or buckwheat substrates, reduced colonization.

Not only colonization, but also saprophytic survival of *Rhizoctonia*, was influenced by the C to N balance of the substrate. Buckwheat and oat stem pieces with C/N ratios, modified by the addition of glucose or $NaNO_3$ to produce a range of C/N ratios 5–120, were buried in GLS and ESL for 56 days. Saprophytic colonization and survival were assayed after 4, 28, and 56 days of incubation. The percentage of colonization of both substrates (Table 4, 5) shows that both saprophytic activity and survival of the colonizing mycelium in the tissues were increased by lowering the C/N ratio of the substrates below those of the controls. Ratios above those of the controls, obtained by adding glucose to the tissues, reduced percentage of colonization and shortened the survival period of *Rhizoctonia*. The results obtained at 56 days of incubation were similar to those obtained at 28 days and are not included in Table 4, 5.

*Spread of R. solani through soil from a colonized substrate.*—Seedling emergence, infection of surviving seedlings, and saprophytic activity, assayed subsequent to seedling removal by the buckwheat method (Table 6), indicated that *R. solani* spread from the colonized

TABLE 2. Effect of length of incubation of 5 *Rhizoctonia* isolates in unsterilized Elsinboro sandy loam on their competitive saprophytic activity in soil

| Isolate no. | Saprophytic colonization[a] at various intervals (weeks) after addition of inoculum to soil | | | |
|---|---|---|---|---|
| | 2 | 9 | 18 | 28 |
| R 1 | 98 | 46 | 19 | 7 |
| R 5 | 100 | 93 | 97 | 74 |
| R 6 | 100 | 98 | 93 | 40 |
| R 7 | 100 | 97 | 84 | 76 |
| R 8 | 100 | 75 | 35 | 40 |

[a]Percentage of buried mature buckwheat stem pieces colonized.

pieces and assumed an active parasitic status in either the presence or absence of competing microflora.

DISCUSSION.—In unsterilized soils, naturally infested with species of *Rhizoctonia*, the greatest saprophytic activity of the pathogen occurred at a wide range of relatively low soil moisture (20-50% MHC). These results agree with those reported by Blair (3), who studied the growth of *R. solani* with glass slides in unsterilized soils; and with those of Das and Western (8), who studied the growth of this pathogen in tubes containing sterilized artificially inoculated soil. The reduction of growth at high soil moisture was attributed by Blair (3) to a decline in soil aeration with an increase in moisture content. High soil moisture would undoubtedly stimulate bacterial activity, which in turn may result in lysis of the fungal mycelium. Kovoor (12) showed that at a moisture level of 30% saturation there was no bacterial action against the hyphae of *R. bataticola* on glass slides buried in soil. At 50% saturation, bacterial attack was perceptible; at 80%, the slides showed bacterial attack at an advanced stage followed by complete lysis of mycelium. On the other hand, accumulation of $CO_2$ in the immediate neighborhood of the substrate in poorly drained soils may prevent or reduce the saprophytic activity of *Rhizoctonia* (unpublished data of the authors).

The greatest saprophytic activity of *Rhizoctonia* in unsterilized soils, as determined by the competitive colonization method in the present experiments, occurred within approximately the same soil moisture

TABLE 3. Effect of C[a] and N[a] enrichment of buckwheat and oat stem pieces on their saprophytic colonization by *Rhizoctonia* in unsterilized greenhouse loamy sand naturally infested with the pathogen

| Supplemental C and N added to the substrate | Saprophytic colonization[b] of stated substrate | |
|---|---|---|
| | Buckwheat stem pieces | Oat stem pieces |
| 1% C | 46 a[c] | 38 a |
| 3% C | 53 ab | 32 a |
| Control | 59 b | 56 b |
| 1% N | 75 c | 64 bc |
| 3% N | 76 c | 72 c |
| 1% C + 3% N | 81 c | 51 b |
| 3% C + 3% N | 83 c | 63 bc |

[a]Supplemental C and N were added by soaking buckwheat or oat stem pieces in glucose or $NaNO_3$ solutions calculated to give an additional 1 or 3% C or N, respectively, on the oven-dry weight basis of the substrates.

[b]Percentage of buried mature buckwheat and oat stem pieces colonized.

[c]Numbers followed by the same letter are not significantly different at the 5% level of probability.

TABLE 4. Effect of C/N ratio of buckwheat stem pieces on their saprophytic colonization by *Rhizoctonia* in greenhouse loamy sand (GLS) and Elsinboro sandy loam (ESL)

| Buckwheat stem piece C/N ratio[a] | Saprophytic colonization[b] at various intervals after substrate addition to stated soil | | | |
|---|---|---|---|---|
| | 4 days | | 28 days | |
| | GLS | ESL | GLS | ESL |
| 5 | 87 | 81 | 47 | 20 |
| 10 | 96 | 87 | 61 | 29 |
| 20 | 90 | 75 | 35 | 27 |
| 30 | 85 | 75 | 44 | 20 |
| 50 | 86 | 74 | 37 | 16 |
| 70 | 83 | 63 | 16 | 21 |
| 95 (Control) | 61 | 61 | 17 | 16 |
| 105 | 59 | 51 | 7 | 17 |
| 120 | 57 | 28 | 1 | 9 |

[a]C/N ratios below and above 95 were obtained by addition of increasing concentrations of $NaNO_3$ and glucose, respectively.

[b]Percentage of buried mature buckwheat stem pieces colonized.

TABLE 5. Effect of C/N ratio of oat stem pieces on their saprophytic colonization by *Rhizoctonia* in greenhouse loamy sand (GLS) and Elsinboro sandy loam (ESL)

| Oat stem piece C/N ratio[a] | Saprophytic colonization[b] at various intervals after substrate addition to stated soil | | | |
|---|---|---|---|---|
| | 4 days | | 28 days | |
| | GLS | ESL | GLS | ESL |
| 5 | 69 | 47 | 47 | 19 |
| 10 | 64 | 44 | 32 | 15 |
| 20 | 52 | 52 | 25 | 8 |
| 30 | 48 | 46 | 19 | 11 |
| 50 | 42 | 47 | 18 | 15 |
| 70 | 39 | 43 | 12 | 7 |
| 83 (Control) | 41 | 41 | 12 | 6 |
| 105 | 45 | 19 | 8 | 4 |
| 120 | 30 | 13 | 7 | 5 |

[a]C/N ratios below and above 83 were obtained by addition of increasing concentrations of $NaNO_3$ and glucose, respectively.

[b]Percentage of buried mature oat stem pieces colonized.

TABLE 6. Spread of *Rhizoctonia solani* (isolate R 7) through soil from colonized buckwheat stem pieces as indicated by radish seedling emergence and infection, and substrate colonization

| Soil treatment and inoculum concentration[a] | Radish emergence (%) | Infection index[b] | Saprophytic colonization[c] (%) |
|---|---|---|---|
| Autoclaved soil | | | |
| 0 pieces | 91 | 0 | 0 |
| 3 pieces | 81 | 2.5 | 89 |
| 30 pieces | 43 | 2.4 | 53 |
| Natural soil | | | |
| 0 pieces | 89 | 0.2 | 3 |
| 3 pieces | 90 | 0.6 | 13 |
| 30 pieces | 64 | 0.9 | 34 |

[a]Low inoculum was provided by 3 colonized buckwheat stem pieces buried 1 cm below the soil surface, near the center of coffee cans containing the equivalent of 1 kg oven-dry soil. High inoculum was provided by mixing 30 colonized buckwheat stem pieces with the equivalent of 1 kg oven-dry soil.

[b]Mean severity rating on surviving individual plants on a scale of 0 (no disease) to 5 (plants completely girdled).

[c]Percentage of colonization of mature buckwheat stem pieces buried in soil subsequent to inoculum and seedling removal.

range in which the maximum parasitic activity was reported by other investigators (2, 17). In addition, Sanford (18) found that even in a fairly dry sterilized soil (19% MHC) at 16°C the pathogen made 5 cm of linear growth in 10 days which would appear adequate for infection of young potato sprouts from a set bearing viable sclerotia. Das and Western (8) found that *Rhizoctonia* root rot of lettuce was more severe on plants grown at 40% saturation capacity than on those grown at 100%.

The relation of temperature to disease development and growth of *R. solani* in sterilized soils and in various culture media has been extensively studied in the past. The effect of soil temperature on the competitive saprophytic activity of the pathogen in unsterilized soil in the absence of a host has received little attention. In the limited experiments of the present work, it is interesting to note that optimum soil temperature for colonization of a substrate differed from 1 soil to another. The discrepancies observed in the optimum temperatures of colonization in GLS, a Maryland soil, and in IFS, a Florida soil, can best be explained by assuming a past natural selection of isolates adapted respectively to moderate and high temperatures. A comprehensive review of the heterogeneity of *R. solani* with respect to the behavior of its isolates at various temperatures has recently been published by Bateman and Dimock (1).

The saprophytic activity of *Rhizoctonia* in soil was not only influenced by soil temperature, moisture, and reaction, but also by the isolates present in soil and by the length of incubation of inoculum in unsterilized soil. This is particularly significant in view of the fact that species of *Rhizoctonia* exist in natural soils as distinct morphological and pathogenic clones of varying saprophytic and parasitic capabilities (unpublished data of the authors). From the data presented in this paper (Table 2) and from a number of other experiments carried out in connection with other phases of the studies on *Rhizoctonia* by the authors, it would appear that heritable characters of each isolate have a definite and decisive place in the active saprophytic colonization of substrates in natural soils. Whether the decline in saprophytic activity of some isolates is concomitant with their elimination from soil or with a transformation to a resistant stage, temporarily inactive, cannot be ascertained at present.

The studies on the effect of length of substrate incubation in soils on the saprophytic activity of the pathogen indicated that the fungus had a brief accelerated activity reaching its peak after 2-4 days (Fig. 1, 2), a period of declining activity between the 4th and 10th day, and a period of equilibrium from the 10th to at least the 120th day. From an ecological viewpoint, this phenomenon may be of great significance. This is especially so since *Rhizoctonia* mycelia and sclerotia were observed microscopically by Boosalis and Scharen (4) in well-decomposed organic debris lying in soil; and since the pathogen can assume a parasitic status from its saprophytic condition in the colonized substrate (Table 6). Availability of rapidly assimilable nutrients in the substrate during the initial stages of colonization may explain the intensive saprophytic activity of *Rhizoctonia* during the initial 4 days. As quickly available food decreases, the saprophytic activity and survival of *Rhizoctonia*, inside the pieces, decline. The decline may not be due only to competition for nutrients, but also to other antagonistic interactions provided by associated microorganisms capable of decomposing resistant plant compounds in the substrate. Absence of an appreciable decline of the saprophytic phase of *Rhizoctonia* in the substrate after the 10th day may be due either to the formation of resting structures (*e.g.*, sclerotia), to the presence of a limited food supply released by the decomposing action of other microorganisms, or to slow utilization of cellulose by *Rhizoctonia* (3).

If competition for nutrients in the foregoing experiments influenced the length of incubation on the substrate, it was not possible to determine the identity of these nutrients. In subsequent experiments, it was shown that P and Ca were not important in affecting the saprophytic activity of the pathogen. Competition for available N on the other hand was involved in all experiments employing buckwheat and oat stem pieces enriched with quickly available N from $NaNO_3$ (Table 3, 4, 5). A decrease of the C/N ratio of the substrate by addition of increasing concentrations of $NaNO_3$ produced a marked increase in the competitive saprophytic activity of *Rhizoctonia* and an increase in the longevity of the active fungus mycelium in the tissue. Loss of viability, on the other hand, was hastened by supplementing the substrate with energy materials (*e.g.*, glucose) that raised the C/N ratios. Without precluding the possibility of a build-up of microorganisms directly antibiotic to or parasitic upon *Rhizoctonia* mycelium, the predominant influence of the substrate microflora must be assigned a competitive role for available N. At high C/N ratios obtained by addition of glucose, an increase in the numbers and activities of colonizing microorganisms may have resulted in N immobilization, and consequently in N deficiency, for *Rhizoctonia*. Nitrogen depletion would decrease not only the initial competitive colonization of the substrate, but also would accelerate the rate of decline of the *Rhizoctonia* mycelium in the colonized substrate. Intensified microbial competition for available nutrients, mainly N, was considered by Garrett (9, 10) as the main cause of decline of *Ophiobolus graminis* in wheat straw. Bacterial competition for available N was also considered by Marshall and Alexander (14) as a significant means of suppressing *Fusarium oxysporum* f. *cubense* in sterilized soil.

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# TEMPERATURE AND MOISTURE REQUIREMENTS FOR GERMINATION OF CONIDIA OF SPHAEROTHECA PANNOSA FROM PEACH

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SUMMARY

The conidia of *Sphaerotheca pannosa* (Wallr.) Lev. var. *persicae* Wor. germinated over a wide temperature range. Slight germination occurred at 4°C. The optimum temperature was from 21-27°C and no germination occurred at 36°C. The most pronounced effect of an optimum temperature was on the rate of germination.

At controlled relative humidity ranging from 43–100%, germination of spores on glass slides was less than 1% whereas 9.5–12.3% of spores on the upper surface of peach leaves germinated.

When the germination chamber containing distilled water was lined with moist filter paper to assure a saturated atmosphere, germination on glass was increased to 2.6% and that on the leaf surface to 16.7%. On leaves under conditions conducive to condensation, 25.7% germination occurred. Spores dusted on parlodion membranes floating on water showed maximum germination equal to that obtained on the leaf surface. Spores at 100% relative humidity on glass slides covered with a parlodion film germinated no better than those on glass alone. Apparently the primary requirement for germination was a suitable source of free water.

Sugars and amino acids were found in water used to wash the upper surface of peach leaves, but this solution had no stimulatory effect on germination.

Powdery mildew fungi are unique: they thrive under dry conditions and the conidia of most species do not require free water for germination. Much variation exists among species regarding moisture requirements for conidial germination. Conidia of *Erysiphe graminis* D.C. (2, 3), *Erysiphe polygony* D.C. (2, 4, 16), and *Uncinula necator* (Schw.) Burr. (5) have germinated at 0% relative humidity under suitable temperature conditions. *Sphaerotheca pannosa* from rose (9), *Erysiphe cichoracearum* D.C. ex Merat (12), *Podosphaera leucotricha* (Ell & Ev.) Salm. (1), and *Sphaerotheca fuliginea* (Schlecht.) Poll. (7) reportedly require conditions of high atmospheric moisture (greater than 95% relative humidity) for optimum germination. Temperature has influenced the tolerance of mildew conidia to moisture stress (3, 5, 12, 16).

Several of the species which require high levels of atmospheric moisture for germination on glass slides germinate on host leaves exposed to low levels of moisture. Conidia of the rose mildew fungus will germinate on leaves exposed to a relative humidity as low as 24% at a temperature of 21°C (9). Rogers (11) found that a relative humidity of 55–100% at the upper leaf surface did not influence the severity of rose mildew until there was condensation on the leaves. Woodward (15) obtained germination of *Podosphaera leucotricha* on the surface of an apple leaf, but not on a moist watch glass. The review by Yarwood (17) presents a more complete discussion of the moisture requirements of powdery mildew fungi.

Yarwood, *et al.* (19), in reviewing the temperature relations of powdery mildews, stated that the optimum temperature for this group of fungi is about 21°C or 4° lower than the average optimum for plant pathogens.

This study was conducted to determine the moisture and temperature requirements for conidial germination of the peach powdery mildew fungus (*S. pannosa* var. *persicae*) in the light of Longrée's (9) results with the form on roses. A 2nd objective was to examine Longrée's hypothesis that the beneficial effect of the host leaf surface upon conidial germination was due to proper relative humidity, as opposed to the interpretation by Yarwood and Hazen (18) that the effect was more likely explained by host stimulation.

MATERIALS AND METHODS.—Unless otherwise indicated, all spores used in the following experiments were produced on Lovell peach seedlings which were held under artificial light at a temperature of 25°C and a relative humidity of about 50%. Spores approximately 1–2 days old were used in all tests. These were obtained by brushing the lesions 24–48 hours before an experiment to remove spores present at that time.

Determinations of temperature requirements for conidial germination were conducted on the upper surface of peach leaves floated on distilled water in open petri dishes. This was necessary because of poor germination on water and on glass slides suspended over water in petri dishes. Due to the lack of lighted temperature control units, germination was determined only in the dark.

A humidity chamber was designed to determine the conidial germination on peach leaves and inert substrates at various levels of atmospheric moisture. The desired levels of atmospheric moisture were maintained by means of saturated solutions of suitable salts (14). The regulating solutions were contained in 4-oz glass jars. Substrates for germination were suspended from the lid, about 1.5 cm above the solution, by a small wooden dowel. The dowel was covered with aluminum foil to prevent absorption of moisture. Leaves were cut into segments about 2.5 cm long. The blade was removed for a distance of 1.5 cm and the midrib inserted into a vial of water through a small hole in a polyethylene plastic membrane. The vial was placed in a hole drilled in the dowel. The leaf segments remained turgid for the 24-hour germination period, although occasional wilting occurred at the lower moisture levels. Segments of glass cover-slips were held in slits cut into the wooden support at the same level as the vial. In order to maintain a constant relative humidity, the jars were submerged in a water bath in which the temperature was maintained at 25.00±0.04°C. The jars were allowed to equilibrate at least 4 hours before each experiment. For comparison, a control, consisting of spores dusted on leaves floating on water in a petri dish, held at 24°C, accompanied each experiment. Segments of the same leaves were used in the jars and as controls.

In all cases, leaves were washed with distilled water and dried before being dusted with conidia. Spores on the inoculated leaf surface were removed by covering the surface with a film of a 2% (w/v using 1:1 ether and absolute ethanol as the solvent) solution of parlodion (5). In about 60 seconds, the film dried forming a transparent membrane in which all particles on the leaf were embedded. The membrane was stripped from the leaf, mounted in lacto-fuchsin, and the percentage of germination was determined.

The parlodion membranes were formed by placing 1 drop of a 5% solution (w/v in amyl acetate) on water in a 9-cm petri dish.

To determine whether organic materials were present on the surface of peach leaves, the upper surface of each of 580 leaves was atomized with 1.5 ml of distilled water at a temperature of 24°C. The leaves were clipped to a large funnel so the run-off could be collected. After the 1st washing, the leaves were placed in a plastic bag for 4 hours and then rewashed, again using 1.5 ml/leaf. The 1st and 2nd washings were kept separate. The run-off was brought to 70% ethanol with 95% redistilled ethanol. The solutions were boiled for 7 minutes, and concentrated to 10 ml under reduced pressure and moderate temperature. The concentrates were analyzed for sugars and amino acids by paper chromatography. For amino acid chromatography, interfering materials were removed by passing the washings through a column of Amberlite cation exchange resin IR 120 ($H^+$). The amino acids were eluted with 4 *N* ammonium hydroxide. The amino acid chromatograms were spotted with 250 $\mu$l of the eluate and developed for 24 hours in butanol-acetic acid-water (6.3:

TABLE 1. Influence of temperature on germination of conidia of powdery mildew of peach

| Temperature (°C) | % germination, Hours after inoculation 6[a] | 12[b] | 24[c] |
|---|---|---|---|
| 4 | <1 | 4 | 8 |
| 12 | 5 | 6 | 22 |
| 15 | 2 | 20 | 28 |
| 18 | 14 | 23 | 30 |
| 21 | 16 | 25 | 36 |
| 24 | 13 | 24 | 35 |
| 27 | 7 | 23 | 34 |
| 30 | 8 | 19 | 19 |
| 33 | 2 | 10 | 6 |
| 36 | 0 | <1 | <1 |

[a] Mean of 2 tests; 1,000 spores counted/temperature.
[b] Mean of 3 tests; 1,800 spores counted/temperature.
[c] Mean of 6 tests; 3,500 spores counted/temperature.

1:2.7), dried, and developed in the 2nd dimension for 24 hours with 80% phenol. Amino acids were visualized by spraying the chromatograms with a ninhydrin reagent.

Sugars were developed for 70 hours with ethanol-butanol-water (1:4:5) and were detected on the chromatogram with ammoniacal silver nitrate (v/v mixture of 1 *N* $AgNO_3$ and concentrated $NH_4OH$). The quantitative determinations were made with the procedure of Dubois, *et al.* (6).

RESULTS.—*Influence of temperature on spore germination.*—The percentage of germination was determined at 6, 12, and 24 hours after the leaves were dusted. Preliminary work showed that nearly maximum germination was obtained in 24 hours. With both Paloro trees and Lovell seedlings, leaf age had no effect on spore germination.

Table 1 shows that spores of *S. pannosa* can germinate over a wide temperature range. During the 1st 6 hours, germination was highest at 18–24°C. At the end of 12 hours, the optimum range was 15–30°C, and after 24 hours, good germination was obtained at 12–30°C, with the optimum at 21–27°C. A small percentage of spores germinated at 4°C and 33°C, but no germination occurred at 36°C. The most striking occurrence of an optimum temperature was at the 6-hour germination period. After 24 hours, germination was high over a wide temperature range.

The data presented in Table 1 are not strictly comparable because more experiments were conducted at the longer time intervals. The large number of spores counted for each temperature, however, should compensate for much of the variation.

The rate of germination at 0–6 hours after inoculation also was determined. In a typical experiment, germination was 1.4, 5.7, and 11.9% after 4, 5, and 6 hours, respectively.

*Moisture requirements for conidial germination.*—1) *Conidial germination on peach leaves and glass slides exposed to various levels of atmospheric moisture.*—The moisture levels tested varied from 100% to 43% relative humidity. Several workers have emphasized the value of using vapor-pressure-deficit (VPD), rather than relative humidity when referring to atmospheric moisture (5, 12). When temperature is constant, however, relative humidity may be used to compare levels of atmospheric moisture. Distilled water was used to obtain a saturated atmosphere and in no case was condensation observed on leaves or glass slips removed from the jars. Spores germinated on the surface of leaves at all moisture levels employed (Table 2). Even on the leaves suspended over distilled water, however, the germination was less than on leaves floating on water in covered petri dishes, used as controls. Condensation was frequently observed on the control leaves. In no case was germination on the glass slips greater than 1%.

Additional tests were made in which jars containing distilled water were lined with moist filter paper to assure a saturated atmosphere. The mean germination obtained in this experiment was 2.6% on the glass slips and 16.7% on leaves in the jars, compared with 22.5% on control leaves. As before, no condensation was observed on leaves or glass in the jars.

Glass slides covered with a parlodion film, Saran Wrap, or aluminum foil showed no better germination than glass alone.

A test also was conducted in which leaves and glass slips were exposed to 99% relative humidity (maintained by 0.5 *M* sucrose) in jars with the slides lined with filter paper. On glass, 1.7% of the spores germinated; on leaves, 19.4%.

Several experiments were conducted in which spores were dusted on microscope slides under conditions where condensation occurred on the glass, or directly on the surface of water. In 2 representative experiments, 8% germination occurred among spores on glass slides on which visible condensation was present

TABLE 2. Germination of conidia of powdery mildew on the upper surface of peach leaves and on glass slips exposed to various levels of atmospheric moisture

| Relative humidity (%) | VPD (mmHg) | Substrate | % germination after 24 hours[a] |
|---|---|---|---|
| 100 | 0.0 | leaf | 12.3[b] |
| | | glass | <1.0 |
| 92 | 1.9 | leaf | 10.8 |
| | | glass | <1.0 |
| 86 | 3.3 | leaf | 12.1 |
| | | glass | <1.0 |
| 75 | 5.9 | leaf | 13.0 |
| | | glass | <1.0 |
| 64 | 8.6 | leaf | 11.0 |
| | | glass | <1.0 |
| 48 | 12.4 | leaf | 9.5 |
| | | glass | <1.0 |
| Control | | leaf | 25.7 |

[a] Mean of 3 experiments; 1,800 spores counted at each moisture level.
[b] The standard error, calculated on the basis of eighteen 100-spore replications, was 1.1, 1.7, 1.2, 1.4, 1.6, and 1.5 for germination on the leaf surface at 100, 93, 86, 75, 64, and 48% relative humidity, respectively.

and 7.7% among spores floating directly on a drop of water.

Germination at the various levels of relative humidity also was determined for the lower leaf surface and was essentially the same as that on the upper surface. This comparison involved 2 experiments with 900 spores counted on each surface at each moisture level. An experiment was conducted to determine whether the leaf surface inoculated had any influence on germination in the control dishes. Percentage of germination of 1,200 spores on each surface was 28.4% on the upper surface and 23.9% on the lower.

2) *Comparison of spore germination on the surface of peach leaves and on parlodion membranes.*—When parlodion dissolved in amyl acetate is placed on water, it spreads to form a thin uniform film which hardens into a membrane as the solvent evaporates. A comparison of rate of germination and total germination was made between spores dusted on peach leaves floating on water in a closed petri dish and spores dusted on parlodion membrane in open dishes. It was necessary to leave the membranes exposed to prevent wetting of the upper surface and consequent reduction in spore germination. At the end of 24 hours, microscopic examination of the membrane surface revealed the presence of small water droplets. Water evaporated through the membranes nearly as rapidly as from a free surface. Germination was allowed to proceed for 24 hours under room conditions (24-26°C).

The total germination on the membranes was essentially the same as that on the leaf surface, but germination was more rapid on the leaf surface. On the upper leaf surface after 6, 12, and 24 hours, germination was 11, 20, and 26%, respectively, whereas on the parlodion membrane for the same period of time 5, 21, and 27% of spores germinated. The germination percentages are the mean values of 2 experiments with 3 replications/experiment and 300 spores counted/replication. A statistical analysis of the data at the 6-hour germination period revealed that the difference in germination was not significant at the 5% level.

To determine whether amyl acetate had any influence on germination, membranes were prepared with parlodion dissolved in ether and absolute ethyl alcohol (v/v). The spore germinations on these membranes were similar to those reported above.

3) *Demonstration of the presence of organic materials naturally present on the surface of peach leaves.*—Considering the suggestion of Yarwood and Hazen (18) that the host provides a germination stimulus, a determination was made of chemical compounds present on the leaf surface.

The upper surface of peach leaves was gently washed and the run-off was analyzed for the presence of amino acids and sugars. Sugars and amino acids were readily detected in the concentrated run-off. The 2nd washing contained a higher concentration of materials than the 1st. On a chromatogram of 250 $\mu$l of the concentrate from the 2nd washing, which represented that material from 15 leaves, the following amino acids were detected and tentatively identified on the basis of standards developed at the same time: aspartic acid, glutamic acid, serine, glycine, asparagine, glutamine, alanine, threonine, gamma amino butyric acid, valine, leucine-isoleucine, proline, tyrosine, lysine, and arginine.

Several silver nitrate positive compounds were detected and quantitative analyses of glucose, sucrose, and fructose were made. The concentrations obtained were 1.4, 1.1, and 1.2 $\mu$g/leaf of glucose, fructose, and sucrose, respectively, in the run-off from washing No. 1; and 2.5, 3.1, and 2.0 for these sugars in the run-off from washing No. 2. These values are the means of 2 determinations. Variation between determinations was less than 0.3 $\mu$g in all cases.

Sugars and amino acids were the only compounds for which analyses were made, but it seems very likely that inorganic salts as well as other organic compounds were present.

Several experiments were conducted to determine whether the leaf washings would influence spore germination. For these tests, washings were collected without the use of alcohol. The washings from 468 leaves were concentrated to a final volume of 10 ml. This concentrated solution and several dilutions were tested in various ways and there was no stimulatory effect on spore germination. These tests, however, are of questionable importance because of the impossibility of duplicating the situation which exists on the leaf surface.

Discussion.—The influence of temperature on germination of *S. pannosa* conidia from peach was very similar to that reported by Longrée (9) for *S. pannosa* from rose. Longrée reported that the optimum temperatures for conidial germination shifted with time to lower temperatures. This was not observed in the present study. After a 6-hour germination period, however, a distinct optimum was observed at 18–24°C. With both forms of the fungus, germination had occurred after 24 hours over a wide temperature range. Temperature could be very important in situations where requirements for germination are favorable for only a limited time.

Germination of peach mildew conidia was less than 3% on glass slides at 100%, 99%, and at certain lower levels of relative humidity maintained under strictly controlled temperature conditions; whereas conidia on leaf segments under identical conditions germinated at 10–20%. Apparently, the leaf provides some factor required for appreciable germination. Conidia on parlodion membranes, floating on water, germinated as well as the maximum germination obtained on leaves. Thus, the factor is not peculiar to the leaf. Further, the same membranes on glass in the jars at 100% relative humidity had no effect on germination. Thus, the parlodion and residual solvent are not stimulatory. This also demonstrates that the low germination on glass was not due to an unsuitable glass surface.

Apparently, a primary requirement for the germination of peach mildew conidia is a suitable source of free moisture. *E. g.*, germination was consistently

higher on leaves under conditions where slight condensation frequently occurred (control leaves) than on leaves in an atmosphere of 100% relative humidity but without condensation (leaves in jar). It appears paradoxical to attribute increased germination to water condensation when it has been shown in this and many other studies (1, 5, 9, 12) that abundant free water is detrimental to powdery mildew conidia. The droplets of water which occur on glandular peach leaves, however, are small and discrete and do not spread to form a continuous film (8). Water droplets occurring on the parlodion membranes also were small and discrete. Rogers' (11) data indicate that continuous water films are more harmful to the development of rose mildew than droplets. The amount of water to which spores are exposed may be critical.

The results on the moisture requirements for spore germination are of interest because of their possible bearing on a controversy in the literature. Longrée found that germination of rose mildew conidia was negligible on glass slides below 95% relative humidity, whereas spores on leaves exposed to 50% relative humidity germinated well. This, coupled with the observation that germination on glass at 96.9–99% relative humidity was equal to that obtained on leaves at the same moisture level, prompted Longrée to conclude that high germination on the leaf was due to suitable relative humidity and not some peculiarity of the leaf. Yarwood and Hazen (18) suggested that Longrée's results were more likely explained by host stimulation, based on their conclusion that the relative humidity on leaf surfaces more nearly approached that of the surrounding atmosphere than that of the intercellular spaces. A similar conclusion regarding microclimate at the leaf surface has been reached by other workers (5, 10, 11, 13).

It is suggested that the spores obtained the required moisture for germination through direct contact with water passing through the cuticle to the surface, possibly through pores or cracks. If so, relative humidity of the air at the leaf surface would not be a major factor influencing conidial germination.

Although it has been demonstrated that organic materials are present on the leaf surface, these compounds may not have any influence on powdery mildew. The evidence presented in this report suggests that water is the primary requirement for conidial germination of peach powdery mildew. The possibility that chemicals from the leaf surface might in some way influence germination and/or subsequent development of the fungus, however, cannot be excluded.

(*Note added in proof*). Recently Keil and Wilson (Plant Disease Reptr. 45:10. 1961) have reported the natural occurrence of *Podosphaera oxyacanthae* (D.C.) d By.) on peach. This points up the difficulty of correctly identifying a powdery mildew in the absence of the perfect stage. In the present study perithecia were never observed either in the field or on plants in the greenhouse. The mildew was identified as *S. pannosa* on the basis of the pannose-type mycelium which was formed.

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## BOOK LISTING

**Monografia Dos Fungos Micropeltaceae.**—*Batista, A. Chaves.*—1959. Publicacao No. 56—Instituto de Micologia, Universidade do Recife (E. Brazil). 519 p.

This is a complete revision of the taxonomy and nomenclature of fungi of the family Micropeltaceae. Included are lists of emended genera, new genera, excluded genera, and new combinations.

# NUTRITION IN RELATION TO THE DEVELOPMENT OF WILTS AND ROOT ROTS INCITED BY FUSARIUM IN RED CLOVER

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SUMMARY

Greenhouse studies on the effects of balanced and unbalanced nutrient solutions on the development of red clover in sand culture with and without *Rhizobium* and with and without *Fusarium oxysporum, F. solani,* or *F. roseum* showed that least disease occurred under conditions optimum for the host. Of 6 concentrations of Hoagland's solution, ranging from 0.05–3.0 times normal, 0.5 was best for host development and least favorable for disease development. The pathogens grew well over the entire range of concentrations tested, but grew best at concentrations somewhat higher than those optimum for the host. Apparently, the effect of concentration on the host was more important than the effect on the pathogens. Histological studies showed that the rate of tissue maturation in roots and stems of red clover increased with concentration of nutrient solution. Tissues formed at low concentrations were succulent and fragile and had relatively little secondary xylem, little lignification, and few fibers. Tissues formed at high concentrations were firm and woody and had better developed vascular elements, more lignification, and abundant fibers. Disease severity usually was highest at concentrations below optimum for the host, but above-optimum concentrations also increased disease. If nitrogen, phosphorus, or potassium was omitted from an otherwise balanced solution, plant development was reduced and disease severity increased. If any of these elements was added to the basal solution, disease severity decreased. Additions of potassium caused the greatest reduction in disease. Plants inoculated with *Rhizobium* consistently were more vigorous and had less disease than uninoculated plants.

The relation of nutrition to disease development has been studied extensively and the literature reviewed by Wingard (18). Many investigators (3, 5, 9, 14) have reported that excess nitrogen predisposes plants to disease, whereas phosphorus, and especially potassium, make plants more resistant. It also has been shown that plant species differ in their responses to specific nutrients and combinations of nutrients, as far as their reactions to pathogens are concerned (8).

Critical work has been done on the relation of nutritional factors to the development of *Fusarium* diseases in cotton (10, 19), tomato (3, 5, 16), and some other crops (13, 15), but not in red clover (*Trifolium pratense* L.). This investigation was made to determine the effects of balanced and unbalanced nutrient solutions on red clover and on the development of the wilts and root rots incited by 3 species of *Fusarium* in this crop. An abstract of some of the results was published in 1959 (1).

MATERIALS AND METHODS.—Nutrient solutions were used in all tests. The basal solution, herein designated as 1 H, was that described by Hoagland and Snyder (6). Six concentrations (0.05, 0.1, 0.5, 1, 2, and 3 H) of this solution were compared. In addition, 2 series of unbalanced solutions were tested. In 1 of these series, additional nitrogen, phosphorus, or potassium was added to the basal solution, in the amounts indicated by Walker and Hooker (17); these solutions are referred to as +N, +P, or +K, respectively. In the other series, nitrogen, phosphorus, or potassium was omitted from the basal solution; these solutions are referred to as −N, −P, or −K, respectively. Trace elements were added to all of the solutions and the osmotic concentrations were made the same by the addition of appropriate amounts of NaCl. In all of the tests, the solutions were adjusted to pH 7.0, but in the greenhouse experiments the pH commonly changed to 6.5–6.8 upon passing through the washed white quartz sand used as the substrate.

All of the experiments concerned with disease development were made in the greenhouse where the plants were grown in sand in 8-in. varnished pots and watered by a continuous drip method with the appropriate nutrient solution. The solutions were allowed to drip at a rate of 800–1,000 ml per day per pot. Holes in the bottom of the pots were plugged with glass wool to retain the sand but permit the excess solution to pass through. Every 3rd day, each pot was flushed with distilled water to remove any accumulation of salts from the bottom of the pots.

All of the plants were of the Lakeland variety and were 3 weeks old when transplanted. The seedlings were produced from clean, surface-disinfected seed in vermiculite kept moist with 0.5 Hoagland's nutrient solution. Seedlings used for transplanting were selected for uniformity in size and vigor. Both nodulated and non-nodulated plants were studied. Nodulated plants were obtained by soaking the seed for 1 hour in a suspension of *Rhizobium trifolii* Dangeard prior to sowing. Temperatures both before and after trans-

planting were 22–24°C, which was near the optimum for the plants (2).

Single-spore isolates of virulent strains of *Fusarium oxysporum* Schlect. emend. Snyd. & Hans., *F. solani* (Mart.) App. & Wr. emend. Snyd. & Hans., and *F. roseum* (Lk.) emend. Snyd. & Hans., from naturally infected red clover roots, were used in the disease studies. Inoculum was increased in potato-dextrose broth on a shaker and was 10 days old when used. Inoculation consisted in mixing 100 ml of a spore suspension, containing approximately 100,000 spores/ml, with the sand in each pot and also dipping the roots of the seedlings in the suspension prior to transplanting. Transplanting immediately followed placing inoculum in the sand. Control plants were treated similarly except that sterile potato-dextrose broth was used instead of the spore suspensions.

All experiments with transplants (transplants were used in all except the histological studies) were terminated 65 days after transplanting; then, the plants were lifted, washed, and critically examined for effects of the treatments. Effects were measured in terms of disease severity ratings and oven-dry weights of the plants. Weights of roots and of above-ground parts of the plants were determined separately.

The effects of the 6 concentrations of the basal solution on the rate of maturation of root and stem tissues of red clover were studied by histological methods. Lakeland red clover seed was sown in vermiculite in 1-gal, self-draining glazed crocks and the substrate kept moist with the appropriate solution for 5 weeks when the plants were harvested. Permanent slides were prepared by killing and fixing the plant materials in an FAA solution, dehydrating, imbedding in paraffin, sectioning, and staining with safranin-fast green.

Effects of the 6 concentrations of the balanced nutrient solution on spore germination and mycelial growth of the pathogens were determined in the laboratory. In these tests, 3% dextrose was added to each solution as a source of carbon. In the germination tests, spores from 3-day-old colonies were transferred to No. 2 cover-slips, each containing a large drop of nutrient solution. A ring of petroleum jelly was placed around the drop on each cover-slip and the latter inverted and placed on a deep-well slide where the jelly caused a tight seal and prevented evaporation. The slides were then placed on glass rods in petri dishes at 24°C and the percentage of germination determined 36 hours later. Approximately 1,000 spores were examined at each concentration in each of 3 tests. In the experiments to determine the effects of the various concentrations on mycelial growth, the fungi were grown in potato-dextrose broth in stationary flasks for 14 days after which the mycelial mats were washed, oven-dried, and weighed. The experiments included 10 replicates and were repeated 3 times.

RESULTS.—*Effects of different concentrations of a balanced nutrient solution.*—1) *On the host.*—Best host development occurred at 0.5 H (Table 1), but good development was obtained at 0.1–1.0 H. Higher and lower concentrations retarded host development. In the optimum range, the plants grew vigorously and their stems became relatively large in diameter; at 2 and 3 H, the plants were smaller, paler in color, distinctly less vigorous, and the stems were smaller in diameter, harder, and more woody; at 0.05 H, the plants were about as tall as at 0.1 H but more succulent and fragile with little stem development. Root development also was affected. Roots produced at 0.05 H were softer and lighter in color than those grown at higher concentrations. As concentration increased, the firmness and amount of cortical browning increased. Nodulated plants were larger and more vigorous than non-nodulated plants at all concentrations, whether or not pathogens were present.

Histological studies of non-nodulated plants showed that the rate of maturation of tissues in roots and stems was slower at low than at high concentrations.

TABLE 1. Effects of 6 concentrations of Hoagland's solution on the development of Lakeland red clover in sand culture with and without *Rhizobium* and with and without *Fusarium* in greenhouse tests at 22-24°C, as indicated by the oven-dry weights of the tops and roots of 86-day-old plants[a]

| Concentration | Nodulation treatment[b] | Avg. oven-dry weight (g/plant) for indicated treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | | *F. roseum* | | *F. oxysporum* | | *F. solani* | |
| | | Tops | Roots | Tops | Roots | Tops | Roots | Tops | Roots |
| 0.05 | None | 1.09 | 0.17 | 0.96 | 0.16 | 0.76 | 0.14 | 0.53 | 0.10 |
| | *Rhizobium* | 1.47 | 0.23 | 1.40 | 0.20 | 1.30 | 0.20 | 0.84 | 0.18 |
| 0.1 | None | 1.11 | 0.25 | 1.10 | 0.22 | 0.97 | 0.22 | 0.86 | 0.21 |
| | *Rhizobium* | 1.57 | 0.30 | 1.45 | 0.29 | 1.38 | 0.28 | 1.07 | 0.23 |
| 0.5 | None | 1.49 | 0.29 | 1.37 | 0.29 | 1.20 | 0.29 | 1.02 | 0.20 |
| | *Rhizobium* | 1.63 | 0.33 | 1.56 | 0.31 | 1.53 | 0.30 | 1.30 | 0.24 |
| 1.0 | None | 1.28 | 0.27 | 1.20 | 0.23 | 0.99 | 0.23 | 0.90 | 0.20 |
| | *Rhizobium* | 1.60 | 0.30 | 1.53 | 0.30 | 1.43 | 0.28 | 1.24 | 0.22 |
| 2.0 | None | 0.94 | 0.18 | 0.78 | 0.14 | 0.76 | 0.15 | 0.51 | 0.14 |
| | *Rhizobium* | 1.09 | 0.19 | 1.00 | 0.17 | 1.00 | 0.17 | 0.97 | 0.16 |
| 3.0 | None | [b] | | | | | | | |
| | *Rhizobium* | 0.78 | 0.12 | 0.75 | 0.12 | 0.73 | 0.10 | 0.63 | 0.09 |

[a]The plants were 3 weeks old at the beginning of each test which lasted 65 days. Data are averages of 2 experiments without *Rhizobium* and 1 experiment with *Rhizobium*. Each trial included 4 replicates of 7 plants/treatment.
[b]No data; this concentration was not included in trials without *Rhizobium*.

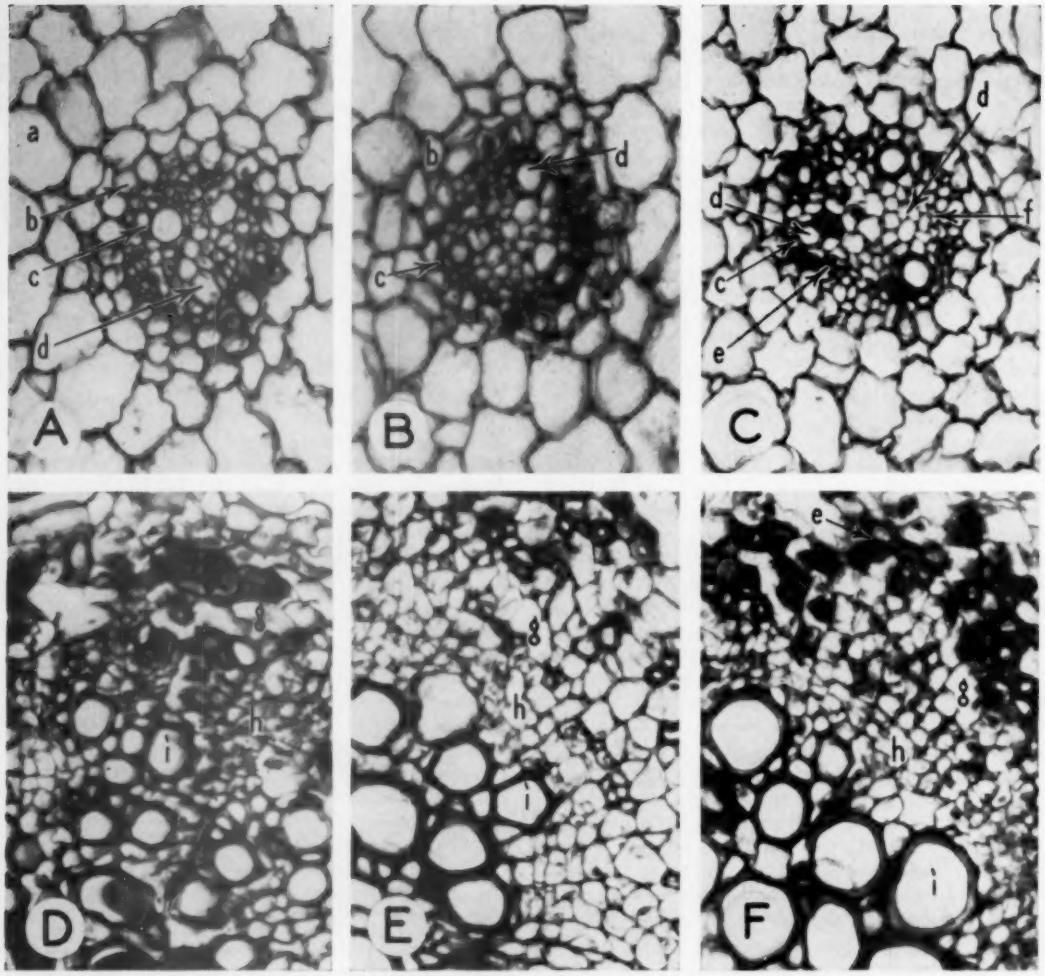


**Fig. 1.** Effects of concentration of Hoagland's solution on maturation of red clover tissues as observed in cross-sections obtained 1 cm (top row) and 15 cm (bottom row) from the root tip. The concentrations of nutrient solution used were 0.1 for **A)** and **D)**, 0.5 for **B)** and **E)**, and 3.0 for **C)** and **F)**. **A)** Protoxylem mature, metaxylem immature, little evidence of fibers. **B)** Similar to **A)**, but metaxylem elements thicker-walled and beginning to mature. **C)** Still greater thickening of some of the metaxylem elements and phloem fibers. **D)** Both secondary xylem and secondary phloem present, few fibers in phloem, narrow cambial zone. **E)** Similar to **D)**, but with greater thickening of xylem elements, more phloem fibers, and a definite cambial zone. **F)** Still greater thickening of xylem elements, many more thick-walled fibers, and wider, active cambium. (a-cortex, b-endodermis, c-protoxylem, d-metaxylem, e-phloem fibers, f-primary phloem, g-secondary phloem, h-cambium, and i-secondary xylem × 200.)

Only 3 concentrations, 0.1, 0.5, and 3 H, will be discussed here.

a) *Root tissues.*—Sections taken 1 cm from the root tip of plants grown at 0.1 H showed few root hairs. Relatively few cells of primary phloem and xylem were present and secondary walls were apparent only in the regions of the protoxylem (Fig. 1-A). In contrast, comparable sections from plants grown at 3 H had many root hairs, more irregular and compressed cortical cells, and more elements of the metaxylem were thick-walled (Fig. 1-C). Thick-walled fibers were apparent in the primary phloem. The rate of tissue maturation at 0.5 H was intermediate between that observed at 0.1 and 3.0 H (Fig. 1-B). A few fibers were evident in the phloem and a few metaxylem elements were maturing.

A comparison of sections taken 15 cm from the root tip also was made. These sections showed the same relative rates of tissue maturation (Fig. 1-D, E, F) as those taken 1 cm from the root tip. At low concentra-

tions, a narrow band of thin-walled cells was present in the cambial zone and abundant secondary phloem and secondary xylem had been formed. These elements were fewer in number and less mature than comparable areas in plants grown at higher concentrations. Some secondary phloem fibers were present underlying the endodermis at all concentrations but the number of fibers and the thickness of their walls increased as the concentration of the nutrient solution increased (Fig. 1-E, F). At the higher concentrations, the cortical cells were more compressed, some were collapsed; the stele consisted of thick-walled primary and secondary xylem elements and rays; and the phloem included numerous thick-walled fibers (Fig. 1-F).

b) *Stem tissues.*—The effects of the various concentrations on the anatomy of the stems (Fig. 2) were similar to those observed in the roots; maturation occurred more rapidly at the higher concentrations. In transverse sections, obtained 1 cm from stem apices of plants grown at low concentrations, lignification was evident only in a few xylem elements. The metaxylem elements were extensive, but mostly thin-walled, and the cambium consisted of a narrow band of thin-walled cells. In contrast, comparable stem sections from plants grown at high concentrations (Fig. 2-C) had proportionally more secondary xylem and the primary vascular elements were lignified and mature in both the protoxylem and metaxylem regions. Some secondary

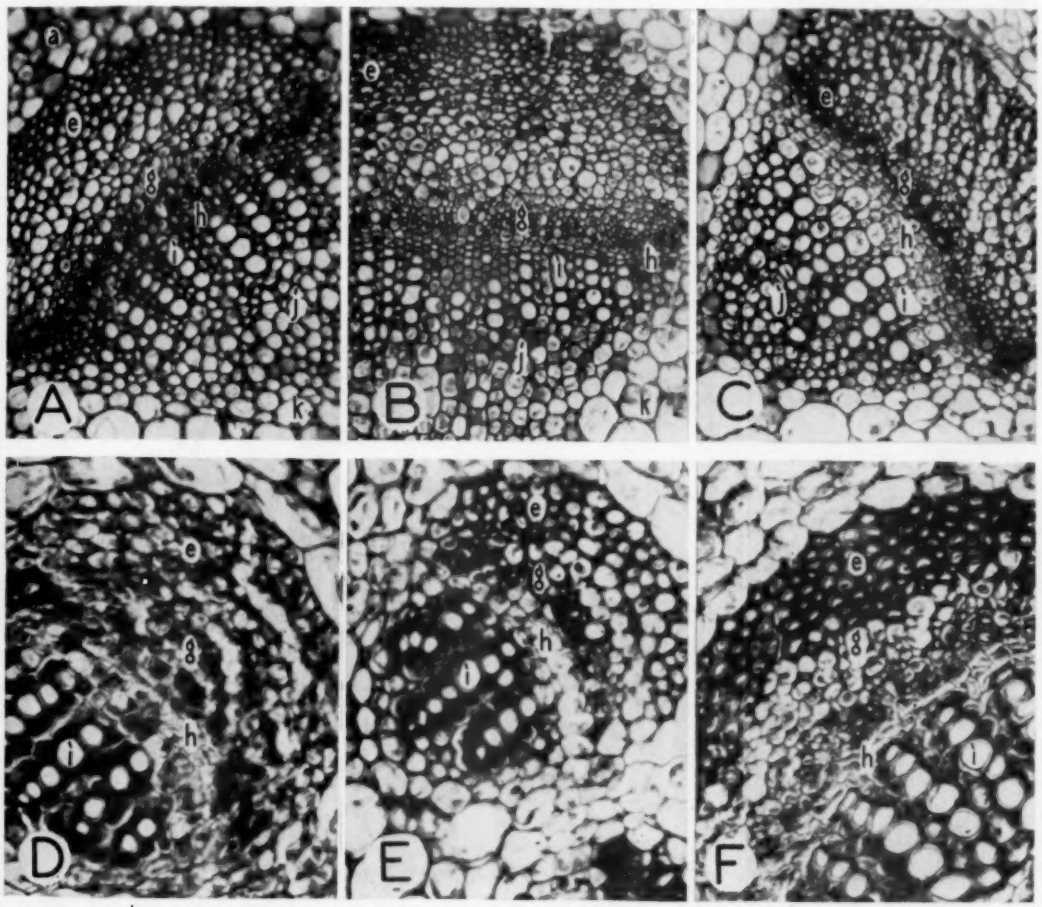


**Fig. 2.** Effects of concentration of Hoagland's solution on maturation of red clover stem tissues as observed in cross-sections obtained 1 cm (top row) and 15 cm (bottom row) from stem apices. The concentrations of nutrient solution used were 0.1 for **A)** and **D)**, 0.5 for **B)** and **E)**, and 3.0 for **C)** and **F)**. **A)** Xylem and phloem present, but only slight thickening in xylem elements; immature fibers in outer phloem; and narrow band of cambium. **B)** Similar to **A)**, except greater thickening of xylem elements; a few thick-walled fibers; and a wider, more regularly arranged cambial band. **C)** Still greater thickening of xylem elements; more mature fibers; and an active cambial band comprised of uniform, compact, regularly arranged cells. Immature secondary xylem elements adjacent to cambium. **D, E, F)** Similar to **A), B)**, and **C)**, respectively, except all tissues more mature. (a-cortex, e-phloem fibers, g-secondary phloem, h-cambium, i-secondary xylem, j-primary xylem, and k-pith × 80.)

phloem and xylem were present. Immature secondary elements were evident adjacent to the active cambium. An abundance of thick-walled phloem fibers was present. When intermediate concentrations were used, the rates of maturation were intermediate (Fig. 2-B).

Stem sections, taken 15 cm from the apices, showed the same relative effects of concentration (Fig. 2-D, E, F) observed closer to the growing point, but as expected the tissues were more highly differentiated.

Sections also were made at the base of the hypocotyl in the transition region of the seedling. At this location, the stem showed a very active cambium and more secondary xylem and phloem than in the root. At high concentrations, fibers were abundant and thick-walled; at low concentrations, these fibers were fewer and showed less thickening.

It was clear from these studies that concentration of a balanced nutrient solution greatly influences the rate of maturation of both root and stem tissues of red clover: very low concentrations retard maturation; higher concentrations hasten it.

2) *On the pathogens.*—The tests made on the effects of the various concentrations on the development of the pathogens prior to infection showed that the fungi studied grew very well over the entire range of 0.05–3.0 H and that their conidia germinated well over this range (Table 2). The percentage of spore germination of *Fusarium oxysporum* increased with concentration up to 2 H. This fungus also produced more mycelial

TABLE 2. Effects of 6 concentrations of Hoagland's nutrient solution on spore germination and mycelial growth of *Fusarium oxysporum* and *F. solani* at 24°C[a]

| Concentration | Spore germination (%) [b] | | Mycelial growth (mg) [c] | |
|---|---|---|---|---|
| | *F. oxysporum* | *F. solani* | *F. oxysporum* | *F. solani* |
| Distilled water | 74 | 78 | [d] | |
| 0.05 | 84 | 94 | 277 | 331 |
| 0.1 | 90 | 97 | 281 | 342 |
| 0.5 | 94 | 98 | 287 | 347 |
| 1.0 | 96 | 98 | 351 | 363 |
| 2.0 | 98[e] | 98 | 351 | 364 |
| 3.0 | 98 | 98 | 350 | 362 |

[a]Data are means of 3 experiments of 10 replicates each.
[b]After 36 hours.
[c]Oven-dry weight after 14 days.
[d]No data.
[e]All data were transformed to arc sin $\sqrt{\bar{X}}$ prior to analysis and Duncan's multiple range test applied to the transformed means. All means not connected by the same vertical line are significantly different at the 5% level. Actual data are given in this table.

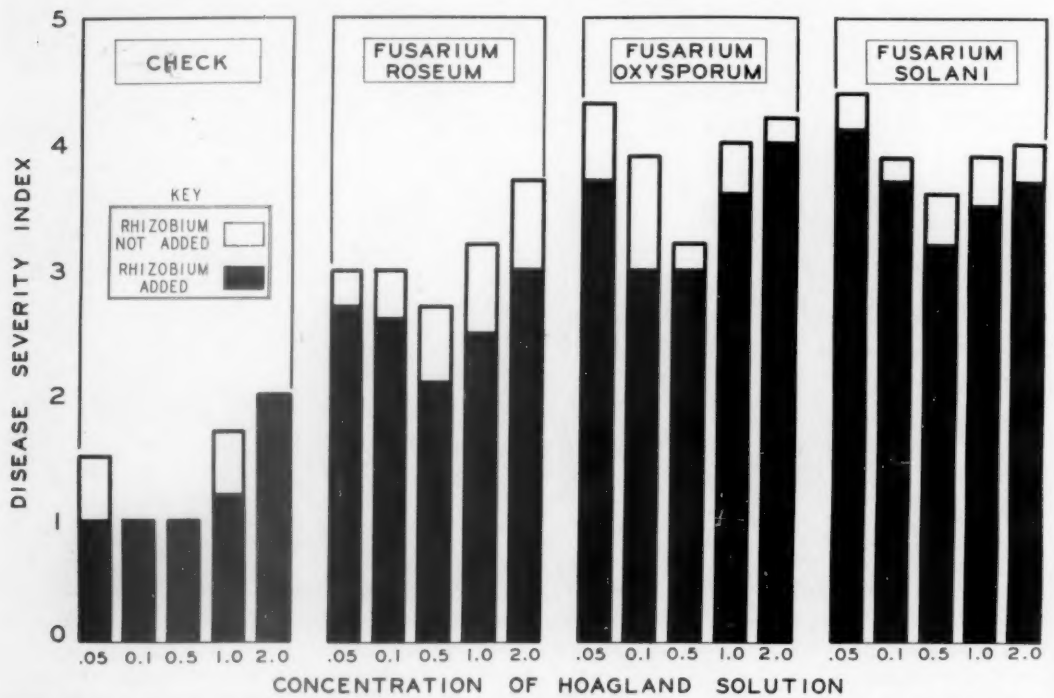


**Fig. 3.** Graphic representation of the effects of 5 concentrations of Hoagland's solution on disease severity of Lakeland red clover, inoculated with *Rhizobium* and uninoculated, when grown in noninfested white silica sand and in sand infested with *F. roseum*, *F. oxysporum*, or *F. solani* for 65 days at 22-24°C. Disease severity index for uninoculated plants is indicated by the total length (black and white sections) of each bar.

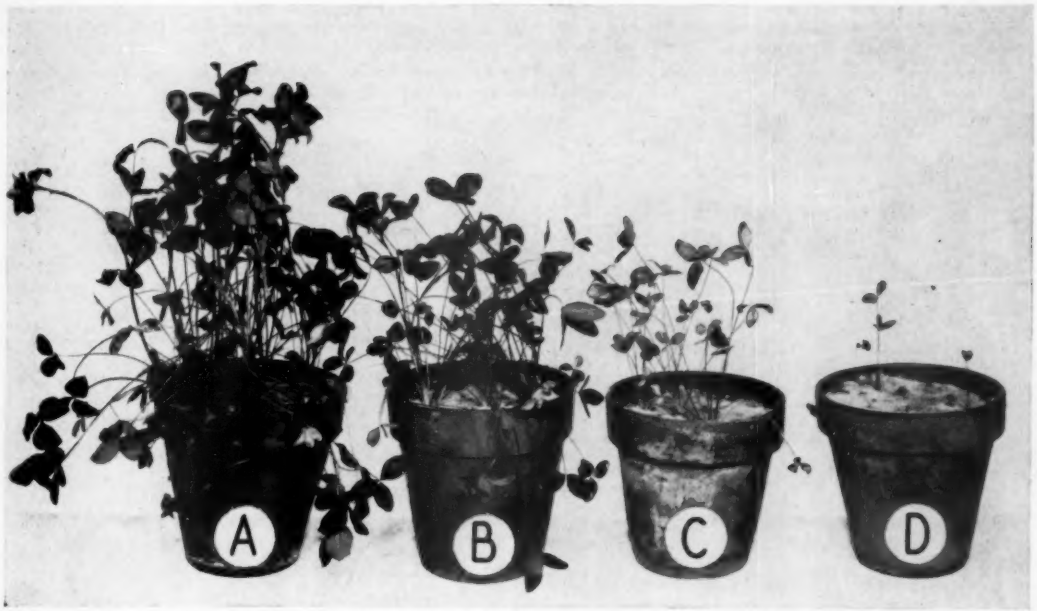


**Fig. 4.** Effects of nitrogen on the development of Lakeland red clover grown in noninfested white silica sand and in sand infested with *F. oxysporum* for 65 days at 22-24°C. **A)** Plants grown in noninfested sand and watered with a complete nutrient solution containing excess nitrogen. **B)** Same as **A)**, in infested sand. **C)** Plants grown in noninfested sand and watered with a complete nutrient solution minus nitrogen. **D)** Same as **C)**, in infested sand. None of these plants inoculated with *Rhizobium*.

growth at 1–3 H than at lower concentrations. *F. solani* responded similarly, but gave higher percentages of germination and more mycelial growth at concentrations below 1 H than did *F. oxysporum*.

3) *On disease development.*—Least disease (Fig. 3, Table 1) developed at 0.5 H, the optimum concentration for host development. Disease severity increased when the plants were grown at higher or lower concentrations and this was true for all 3 of the pathogens studied. Nodulated plants were more vigorous and had less disease at all concentrations than non-nodulated plants.

*Effects of unbalanced nutrient solutions.*—1) *On the host.*—Increases of nitrogen above the amounts contained in the basal solution increased the dry weights of red clover plants whether inoculated with a pathogen or not and whether *Rhizobium* was present or not (Table 3). Conversely, when nitrogen was omitted the dry weights of the plants were greatly reduced (Fig. 4). The effects on dry weight of increases or deficiencies of phosphorus were similar to the corresponding effects of nitrogen but smaller in magnitude. Adding potassium to the basal solution increased the dry weight of the plants only when *Rhizobium* was absent, and then only slightly. Omitting potassium decreased plant growth in most cases.

Diseased plants did not always respond in the same way as healthy plants. For example, plants infected with *Fusarium solani* showed no increase in weight from adding phosphorus to the basal solution whereas uninfected plants benefited from additional phosphorus. In all of these tests, plants inoculated with *Rhizobium* were larger and more vigorous than uninoculated plants.

2) *On disease development.*—Disease severity increased when nitrogen, phosphorus, or potassium was excluded from the basal solution and decreased when any of these elements was added to the basal solution (Fig. 5). Additions of potassium caused the greatest reductions in disease. Plants inoculated with *Rhizobium* had less disease than uninoculated plants.

On the assumption that the form of nitrogen might influence disease severity, a comparison was made of the effects of ammonium ions and nitrate ions. In this test, the nitrate solution was the same as normal Hoagland's solution. In the ammonium solution, the calcium nitrate and potassium nitrate were replaced, respectively, by calcium chloride and potassium chloride; and an equal amount of nitrogen was added in the form of ammonium chloride. Growth of the clover plants was better when they were given nitrate nitrogen, but form of nitrogen had no apparent effect on disease severity.

DISCUSSION.—Host nutrition was an important factor in the development of root rots and wilts incited by *Fusarium* spp. in red clover. It affected both the rate of development and the ultimate severity of these diseases. Least disease occurred at nutrient levels most favorable for the host. Since the pathogens grew very well at these levels, it was concluded that the

TABLE 3. Effects of high and low amounts of nitrogen, phosphorus, and potassium on the development of Lakeland red clover in sand culture with and without *Rhizobium* and with and without *Fusarium* in greenhouse tests at 22-24°C, as indicated by the oven-dry weights of the tops and roots of 85-day-old plants[a]

| Nutrient solution[b] | Nodulation treatment | Avg. oven-dry weight (g/plant) for indicated treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | | *F. roseum* | | *F. oxysporum* | | *F. solani* | |
| | | Tops | Roots | Tops | Roots | Tops | Roots | Tops | Roots |
| Basal | None | 1.28 | 0.22 | 1.20 | 0.20 | 0.93 | 0.13 | 0.90 | 0.20 |
| | *Rhizobium* | 1.71 | 0.34 | 1.63 | 0.31 | 1.44 | 0.26 | 1.24 | 0.22 |
| Basal + N | None | 1.40 | 0.23 | 1.29 | 0.23 | 1.07 | 0.22 | 0.93 | 0.21 |
| | *Rhizobium* | 1.83 | 0.36 | 1.78 | 0.29 | 1.60 | 0.28 | 1.30 | 0.22 |
| Basal − N | None | 0.47 | 0.12 | 0.42 | 0.12 | 0.35 | 0.09 | 0.35 | 0.07 |
| | *Rhizobium* | 0.75 | 0.14 | 0.57 | 0.13 | 0.50 | 0.12 | 0.41 | 0.09 |
| Basal + P | None | 1.37 | 0.27 | 1.25 | 0.24 | 1.23 | 0.20 | 0.75 | 0.13 |
| | *Rhizobium* | 1.77 | 0.31 | 1.63 | 0.28 | 1.56 | 0.23 | 1.20 | 0.21 |
| Basal − P | None | 1.05 | 0.21 | 0.84 | 0.16 | 0.83 | 0.16 | 0.64 | 0.14 |
| | *Rhizobium* | 1.26 | 0.25 | 1.13 | 0.21 | 1.00 | 0.17 | 0.90 | 0.14 |
| Basal + K | None | 1.32 | 0.24 | 1.21 | 0.24 | 1.14 | 0.24 | 1.02 | 0.21 |
| | *Rhizobium* | 1.62 | 0.25 | 1.58 | 0.24 | 1.32 | 0.27 | 1.25 | 0.22 |
| Basal − K | None | 1.11 | 0.23 | 1.07 | 0.23 | 0.97 | 0.20 | 0.86 | 0.13 |
| | *Rhizobium* | 1.58 | 0.25 | 1.38 | 0.24 | 1.23 | 0.22 | 1.19 | 0.20 |

[a]The plants were 3 weeks old at the beginning of each test which lasted 65 days. Data are averages of 2 experiments without *Rhizobium* and 1 experiment with *Rhizobium*. Each trial included 4 replicates of 7 plants/treatment.
[b]A 1.0 concentration of Hoagland's solution was used as a basal solution and N, P, or K was added in additional amounts or completely omitted from this basal solution.

principal effects of the various elements and combinations of elements were on the host. Below- or above-optimum levels for the host increased susceptibility. Plants lacking vigor were more susceptible than vigorous ones. Obviously, many factors influence vigor but nutrition definitely is 1 of these factors. Nodulated plants were consistently more vigorous and had less disease than non-nodulated plants.

The effects of low and high concentrations of balanced nutrient solutions on red clover were similar to those described for sweet pea (12). At low concentrations, the plants grew vigorously, were succulent, and green; both the roots and tops had a high proportion of young active cells containing dense protoplasm. All tissues differentiated and matured slowly. At high concentrations, the plants grew less vigorously, were less succulent, and were pale. Tissues differentiated and matured more rapidly. Both the roots and tops had relatively low proportions of young cells with dense protoplasmic contents.

Of the 6 concentrations of Hoagland's solution compared, 0.5 H produced the best plants. When nitrogen, phosphorus, or potassium was omitted from an otherwise balanced solution, the growth of the plants was reduced. Added amounts of nitrogen, phosphorus, and in some cases potassium, in the basal solution, increased plant development, showing that the basal solution (1 H) was somewhat deficient in these elements.

The nutrient solutions used caused a trace of tan to brown root discoloration (Fig. 3, 5) which increased in intensity with increases in salt concentration or temperature. This discoloration was due to the salts and not to microorganisms. Each of the 3 *Fusarium* species studied incited both wilt and cortical rotting of the roots; *F. oxysporum* incited mostly wilt symptoms, whereas *F. solani* and *F. roseum* caused mostly cortical rots. Both kinds of symptoms were considered in assessing disease severity.

The nature of the greater susceptibility of plants grown in solutions below- or above-optimum for the host was not ascertained. It would seem logical that the thin-walled, immature, unlignified cells produced at low concentrations would be more readily entered and destroyed by pathogens than the thicker-walled, more mature, lignified cells produced at higher concentrations. It also is possible that cells grown under different nutrient conditions differ chemically and physiologically and that such differences influence susceptibility. Tissues produced at above-optimum salt concentrations would appear better able, from a morphological standpoint, to resist pathogens than tissues produced at optimum concentrations. Since this was not the case, factors other than morphology must play some role.

Although the intake of nutrients may occur freely with a fairly high concentration of salts in a nutrient solution (7), water absorption is markedly reduced (12). This probably contributes to the early loss of protoplasm in many of the cells produced at high concentrations. High salt concentrations also may have a marked effect on plant metabolism even when salts are not sufficiently concentrated to cause plasmolysis of the root hairs. In apple (11) and many other perennial plants (4), the initial stages of protein synthesis occur almost exclusively in the fine succulent rootlets. Roots, however, lose their capacity for protein synthesis when grown in high concentrations of salts. Nitrate absorption is not limited (12), but the roots are unable to assimilate nitrate, that is, synthesize amino acids and proteins. Therefore, the effect is nitrogen deficiency. Further work is required before the nature

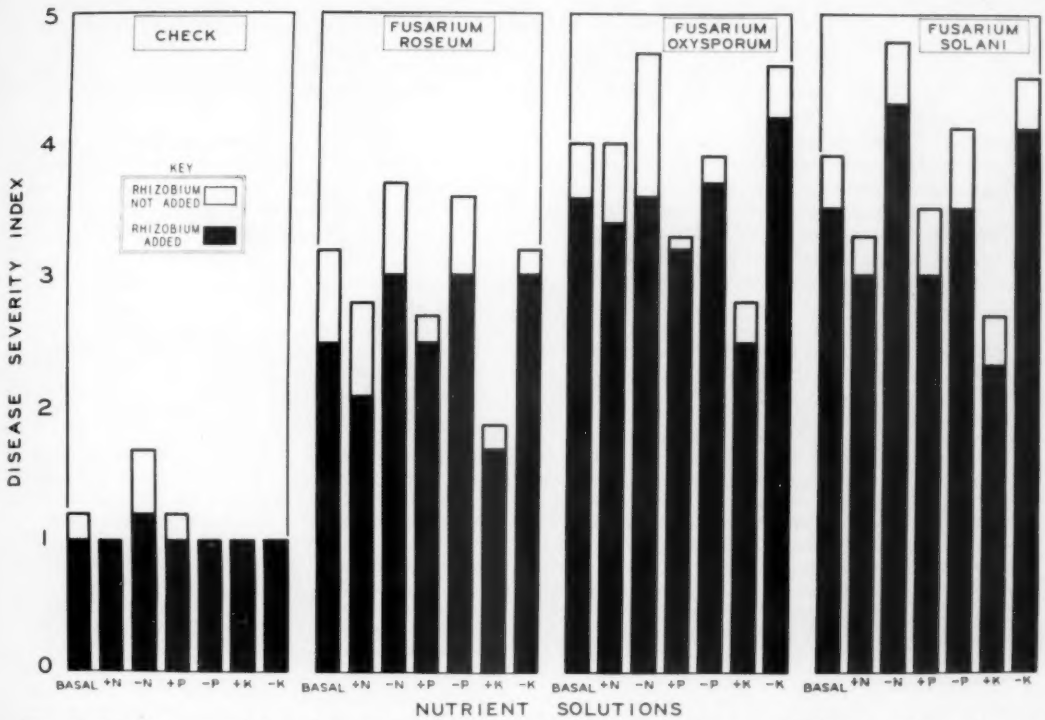


**Fig. 5.** Graphic representation of the effects of high and low amounts of nitrogen, phosphorus, and potassium on disease severity of Lakeland red clover, inoculated with *Rhizobium* and uninoculated, when grown in noninfested white silica sand and in sand infested with *Fusarium roseum*, *F. oxysporum*, or *F. solani* for 65 days at 22-24°C. Disease severity index for uninoculated plants is indicated by the total length (black and white sections) of each bar.

of resistance to root rots and wilts in red clover can be fully explained.

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# SALT INJURY TO TREES

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SUMMARY

A 7-year test on applications of sodium chloride (NaCl) and calcium chloride ($CaCl_2$) to the sites of maple, oak, hickory, black birch, ash, and white pine indicates that winter road salting probably does no great harm to trees in Massachusetts.

A sugar maple died after 186 consecutive weekly applications of NaCl, each at 50 T/acre, or 10 lb on the soil within a circle of 12-ft radius from the trunk, a total of 1,860 lb. Chloride (Cl) concentration in the leaves of this tree was 73 times, and in twigs 13 times, that in nonsalted check maples. Other maples, a birch, and a small pine had died by the end of 201 similar applications of NaCl over their roots, but a white oak so treated showed no injury. A red maple bore stunted yellow foliage after 201 such applications of $CaCl_2$ over its roots, but a black oak so treated appeared normal. The leaves and twigs of the treated oaks contained about the same amount of Cl as those of untreated oaks; much less than those of the other trees in the treated plots.

Sugar maples which received run-off water from adjacent road surfaces salted with NaCl or with $CaCl_2$ at 50 T/acre per treatment, 15 times at weekly intervals during each of 6 winters (total, 75 applications), contained 3–6 times as much Cl in their leaves as check maples, but no more in their twigs. The trees treated only in winter showed no symptoms of injury. Evidently, the Cl from the soil was accumulated in the leaves against a gradient and was removed from the trees when the leaves fell each autumn.

INTRODUCTION.—Salting to control dust in summer on dirt roads is still practiced occasionally; in northern states, highway departments regularly apply salt and sand mixtures to icy roads in winter. Injury from the salt in these mixtures has been regarded widely as a major cause of unthriftiness in shade trees along streets (9, 26). Symptoms ranging from decreased annual growth to dying back of branches have been attributed to salt injury. Such symptoms, however, may be caused by other factors, such as drought; girdling by roots; pavement over roots; decay, wilt, or canker diseases; insect or nematode attacks; altered soil level or water table; gas leaks; compacted soil; injury caused by construction; and improper transplanting.

Pathologists often cannot decide whether salt applications contribute seriously to deterioration of particular street trees. Trees far from any salting frequently have the same symptoms as those near salted roads. Soil tests for soluble salt content and leaf or twig tests for chloride (Cl) content seldom reveal high enough levels to indicate that salt injury caused decline. Indeed, small amounts of salt have beneficial effects on certain plants, through liberation of K and deflocculation of soil particles (24), and even on plants grown in nutrient solutions (8). Yet, dead trees often surround storage piles of salt and sand mixture along highways.

A long-term project was undertaken in 1952 in Massachusetts to study shade tree injury from salt applications to roads or to soil around trees and to establish diagnostic criteria for such injury.

REVIEW OF LITERATURE.—There is substantial evidence that excess salt can harm trees. Little has been published, however, about the relationship between particular symptoms of shade tree injury and particular amounts of Cl in the tissues or particular amounts of salt in the soil around the roots of given shade tree species. One usually must extrapolate from results of work with orchard trees.

Scorch symptoms have occurred on pecan leaves containing 1% or more Cl by dry weight. Cl content of the soil beneath these trees exceeded 200 ppm. The trees were damaged when Cl content of the leaves exceeded 0.6% (10). Tip burn occurred on avocado leaves containing 0.5% or more Cl (5–10 times that in normal leaves) in California (2). In Texas, all tested avocado leaves with tip burn contained 0.22–1.48% of dry weight as Cl, whereas all without symptoms contained 0.07–0.23% (7). Injured peach leaves contained about 2% Cl, whereas uninjured checks contained much less than 1% (12).

A lethal level of Cl accumulation in stone fruit and almond trees is 50–60 meq/100 g dry weight, which is 1.8–2.1% (3). The limits of Cl content before foliage injury appeared were 1% of dry weight for apricot and peach, 0.6% for prune and plum, and 1.2–1.8% for almond (4).

Dying leaves on broadleaved trees inundated by tidal waters after the 1938 hurricane contained 10 times the concentration of Cl found in living leaves on the same trees or in leaves on unaffected trees (29).

Oak seedlings grew, although poorly, in soil treated with 0.2 *M* NaCl, which gave a salt concentration of 0.44% of "completely dry weight of soil" (20). Stunted growth of oak seedlings was evident at 25% of this concentration.

Single applications of KCl at 214 lb/acre (which would produce a soluble salt content in the soil of more than 100 ppm) caused little damage to pine, fir, or larch seedlings growing in sand, but injury did occur at higher rates (17). Scotch pines died in soil with Cl content 6 times the normal level; injured needles contained more than 50% more Cl than needles of control trees (28). Application of $CaCl_2$ at 12.5 T/acre destroyed all vegetation, although soil so treated supported a good crop of barley 1 year later (30).

Recent literature on effects of salt in the soil upon plant growth has been thoroughly reviewed by Hayward and Bernstein (11), and earlier literature was covered in the review articles which they cite.

Materials and Methods.—The 1st salt application of a long series was made on December 10, 1952. Each application was at the rate of 0.05% of the dry weight of a 6-in. layer of soil, *i.e.*, at a rate of about 50 T of salt/acre. The fine or very fine sandy loam in all the plots is of glacial windblown origin (6).

Altogether, 15 applications of either $CaCl_2$ flakes or commercial NaCl were made during the same winter to each of 3 road stretches (called "run-off plots") and to each of 3 areas of soil directly over the roots of trees (called "direct-application plots"). No applications were made between March 18, 1953, and December 23, 1954. On the latter date, treatments were resumed.

*Run-off plots.*—Fifteen treatments were made during each of the next 6 winters, from mid-December until the end of March, to each of the 3 run-off plots, a total of 90 additional applications (105 in all) to each location.

A 160-ft stretch of paved road was chosen for applications of $CaCl_2$ at the rate of 24 lb/week. This road had been banked on a curve so that run-off from the hard surface drained on the soil around the trees of a mixed hardwood forest. The maples in this plot grew 10–15 ft from the road edge on the inner side of the curve. The soil here was well drained, with a 3-in. layer of soil high in organic matter at the top; then, 26 in. of fine sandy loam; and beneath that, loamy sand and gravel.

An adjoining 120-ft portion of the same paved road, similarly banked, received 18 lb of NaCl weekly. The roadside soil here had 3 in. of coarse road wash at the top; then, 29 in. of very fine sandy loam; and beneath that, fine gravel and coarse sand. Trees in this plot grew approximately 10 ft from the road edge.

A 120-ft stretch of dirt road, with sufficient slope to ensure run-off to 1 side, received 18 lb of NaCl weekly. The soil beside this road was excellently drained, with 4 in. of coarse road wash at the top; then, 4 in. of fine sandy loam; and beneath that, loamy sand and gravel. Many trees, including a sugar maple (*Acer saccharum* Marsh.) 8 in. in diameter at breast height (dbh) grew in the soil on which the run-off flowed from this dirt road. Trees in this plot grew 3–4 ft from the road edge.

*Direct-application plots.*—Weekly treatments were made throughout the year to each of the 3 direct-application plots from December 23, 1954, through July 11, 1958, a total of 186 additional applications (201 in all) to each location.

A group of 3 trees—red maple (*Acer rubrum* L.) 8-in. dbh, American elm (*Ulmus americana* L.) 14-in. dbh, and black oak (*Quercus velutina* Lam.) 11-in. dbh—received 10 lb/week of $CaCl_2$ over their roots within a single circle of 12-ft radius. The soil here was excellently drained, with a 3-in. layer high in organic matter at the top; then, 16 in. of fine sandy loam; and beneath that, loamy sand and gravel.

One hemlock [*Tsuga canadensis* (L.)] Carr. received 10 lb/week of NaCl over its roots within a circle of 12-ft radius during the winter of 1952–53. Because the salt leached away rapidly from the sandy soil beneath this tree, a sugar maple with 2 trunks (5-in. and 6-in. dbh), growing in a heavier soil, substituted for the hemlock for the 186 salt applications of 1954–58. The drainage of the soil here was good; the top 2 in. were high in organic matter; the next 22 in. consisted of very fine sandy loam; and the subsoil, of loamy sand and fine gravel.

A group of 7 trees—1 sugar maple 3-in. dbh, 1 white oak (*Quercus alba* L.) 5-in. dbh, 1 ash (*Fraxinus* sp.) 5-in. dbh, 1 black birch (*Betula nigra* L.) 4-in. dbh, 1 white pine (*Pinus strobus* L.) 2-in. dbh, and 2 pignut hickories (*Carya glabra* (Mill.) Sweet) 3-in. and 4-in. dbh—received 7 lb/week of NaCl over their roots in a single approximately rectangular area 10 x 30 ft. The drainage of this soil was good; the top 5-in. layer was coarse road wash; the next 10 in. consisted of very fine sandy loam with a few small stones; and the subsoil was loamy gravel.

*Analytical methods.*—The soluble salt content of the soil, reported as ppm of the air-dry weight of the soil, was determined by measuring conductivity with a "Solubridge," by Mr. E. F. Vlach and Prof. B. Gersten of the Soil Testing Laboratory, University of Massachusetts.

The chloride contents of the leaves and twigs, reported as percentages of their air-dry weights, were determined by drying and pulverizing them, reducing them to ashes under low heat, and precipitating the chlorides with a standard solution of silver nitrate. Leaves were collected for chloride analysis before autumn coloration appeared in 1958. Dormant twigs were collected for this purpose after normal autumn defoliation. The leaf and twig chloride determinations were made by Mr. D. F. Owen of the Feed, Fertilizer, and Dairy Law Control Services, University of Massachusetts.

Results.—At the end of the 1st winter of salting, no injury was detected in the foliage of any treated tree (19). Soluble salt content of the soil, which was less than 10 ppm in all plots before the experiment,

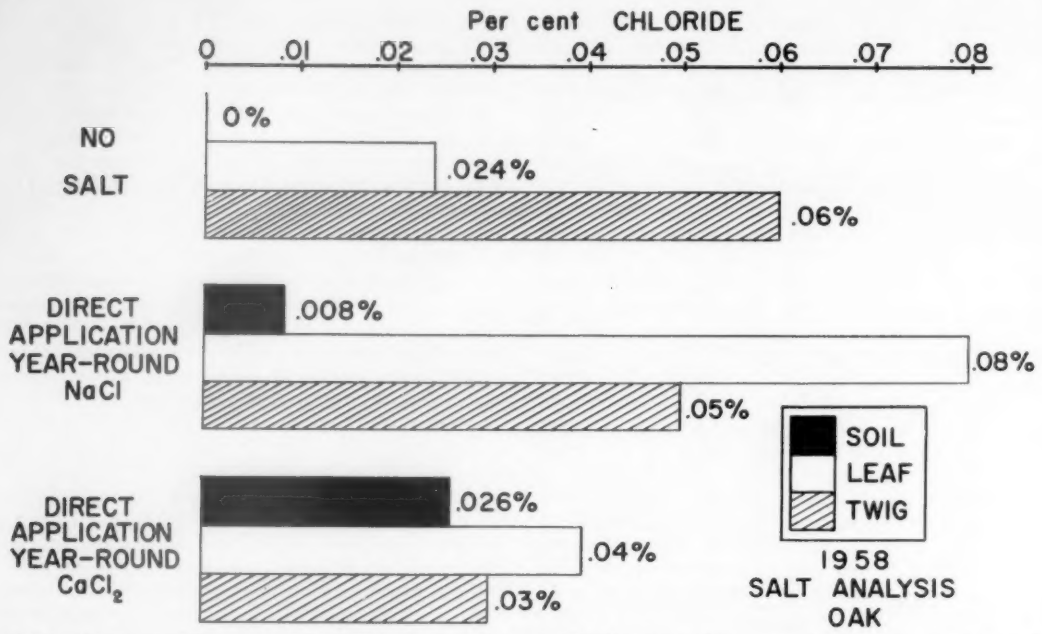


**Fig. 1.** Concentration of chloride in soil, leaves, and twigs of oaks after 6-year treatments with 2 different salts applied in the same manner. Note that the scale (percentage of chloride) is much expanded compared with that of **Fig. 2.**

varied 0–70 ppm in the various plots in May, 1953. In December, 1954, before salting was resumed, no soluble salt could be detected in soil of any of the plots.

*Run-off plots.*—In the run-off plots at the end of the 2nd winter of treatment (spring of 1955), no symptoms of injury were detected on any of the treated trees, but the grass beneath them had died and the other small vegetation was noticeably sparser. The soluble salt contents of the soils in the run-off plots in July, 1955, were 6–23 ppm.

No symptoms of injury were observed on any of the trees in the run-off plots after 7 winters of salt application to adjacent road surfaces, or on any of the untreated check trees. Cl contents of their leaves and twigs did not differ appreciably (Table 1). During the course of the experiment, highway crews removed many of the large maples nearest to the paved road to improve visibility on its curve, but left numerous seedlings and several large maples in the drainage ditch, where they received the run-off from the road surface.

*Direct-application plots.*—In the direct-application plots in the spring of 1955, all grass and small herbaceous plants had died under the treated trees. The soluble salt contents of the soils of these plots in July were 110–314 ppm. The maple leaves were somewhat cup shaped, with necrotic margins and chlorotic interveinal tissue. A thin yellow band separated the dead portions from the green portions. The leaves of oak, elm, and birch also were somewhat chlorotic between the veins, and the tips of the pine needles had yellowed and died (14). Wallace and Moss (29) and Viro (28) also observed yellowness of pine foliage injured by salt. The red color reported on needles of Norway spruces on the sides facing a road treated with $CaCl_2$ (27), and on needles of pine trees beneath which barrels of $CaCl_2$ had been stored (25), was not detected here.

In October, 1955, the maple with 2 trunks (5-in. and 6-in. dbh) in heavy soil, treated throughout the preceding year with 10 lb of NaCl weekly, lost its foliage before the neighboring trees did. By July, 1958, this maple was nearly dead, and its trunks had been entered by boring insects. Only a few branches still bore leaves, all of which had severe "scorch" symptoms (15). Direct-application treatments then were stopped, after nearly 4 years of consecutive weekly applications, during which nearly 1 T of rock salt had been applied directly over the root system of this tree.

The soluble salt concentration in the soil around the dying maple's roots in July, 1958 was 800 ppm, compared with 0–15 ppm in soil under symptomless maples that had received winter run-off salting only. The Cl concentration in the remaining foliage of the dying maple was 3.64%, compared with 0.05–0.29% in the foliage of the symptomless maples (Fig. 2). The Cl concentration in twigs after leaves fell in the autumn was 0.63% for the dying maple compared with 0.05% for symptomless maples that received run-off salting in winter only and 0.05% for an untreated maple (16).

Thus, the concentration of Cl in the foliage of the

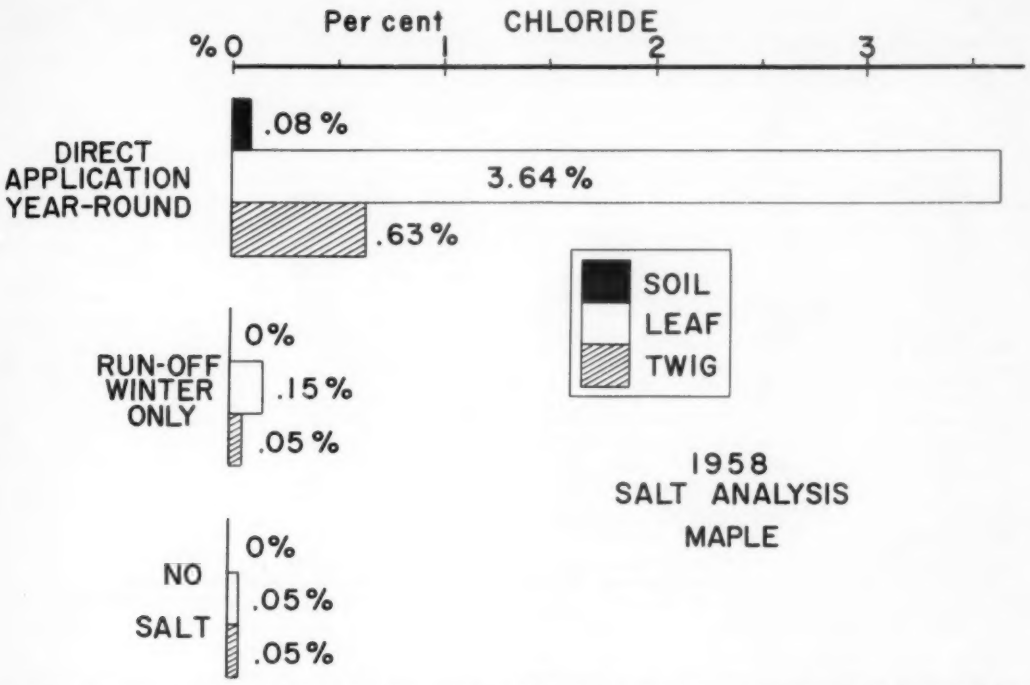


**Fig. 2.** Concentration of chloride in soil, leaves, and twigs of maples after 6-year treatments with NaCl applied in 2 different manners. Note that the scale (percentage of chloride) is much condensed compared with that of **Fig. 1.**

dying maple in this direct-application plot was 13–73 times that in foliage of maples in the run-off plots, 73 times that in foliage of untreated maples, 6 times that in its own twigs, and 45 times that in the soil around its own roots.

A year after the tree's death, however, Cl in twigs was only 0.11%, hardly more than that in check trees.

Table 1 shows the conditions of the trees in all plots and the concentrations of salt in the soil around their root systems 1 week after year-round salting stopped (July, 1958), of Cl in their leaves shortly before autumn color appeared (August, 1958), and of Cl in their twigs after the leaves fell (November, 1958).

Half of the trees in the direct-application plots died during the course of the experiment. The 2 sugar maples, the birch, the pine, 1 hickory, the ash, and the elm had died and had been removed before leaf and twig Cl analyses were arranged.

Discussion.—No symptoms of injury have yet been observed on trees where salt was applied only in winter. Since the ground was frozen during the period of this salting, most of the salt probably dissolved in melted snow and ran off along the surface without reaching any tree roots. Analyses of soil collected in spring or summer from the plots salted in winter only, often revealed little or no increase in soluble salts above the level of the check plots.

Nevertheless, some salt must have entered the topmost layers of the soil even in the run-off plots, to which salt was applied only in winter, because the grass died during the 1st few years of the test along the downhill edge of the salted parts of each road. No young grass plants grew there during the remainder of the experiment. In all direct-application areas, to which salt was applied weekly throughout the year, the grass died during the 1st full year of treatment. Evidently, dying of grass should be sought where salt injury to trees is suspected.

Most of the salt that remained near the trees when the ground thawed probably leached away before the foliage expanded, and hence before transpiration requirements promoted the intake of much water by the roots. Probably, the trees are no less susceptible to salt injury in cool weather than in warm. Research with rice (21) indicates that decreased salt injury at lower temperatures is the result not of decreased plant susceptibility but of decreased intake of water (and salt), because of decreased transpiration in cooler air.

Another indication that at least some of the salt from the applications made only during the winter entered the soil is that the foliage of trees in the run-off plots contained up to 6 times as much chloride as foliage of untreated check trees (Table 1). Dormant twigs of the run-off plot trees, however, contained no more Cl than twigs of check trees.

A similar contrast in Cl content, between foliage and dormant twigs, occurred in the direct-application plots treated throughout the year (Fig. 2). Apparently,

TABLE 1. Tree condition, chloride contents of leaves and twigs, and soluble salt contents of soil in plots treated with sodium chloride or calcium chloride for 6.5 years; analyses as percentages of dry weight

| Method of treatment | Kind of salt | Tree species | Tree condition in 1958[a] | Soil soluble salts (July) | Foliar chloride (Aug.)[c] | Twig chloride (Nov.)[c] |
|---|---|---|---|---|---|---|
| Direct[d] | NaCl | sugar maple | dying | 0.08 | 3.64 | 0.63 |
| Direct[d] | NaCl | sugar maple | dead | 0.01[b] | | |
| Direct[d] | NaCl | sugar maple | dead | 0.01[b] | | |
| Direct[d] | NaCl | white oak | ns | 0.01[b] | 0.08 | 0.05 |
| Direct[d] | NaCl | black birch | dead | 0.01[b] | | |
| Direct[d] | NaCl | white pine | dead | 0.01[b] | | |
| Direct[d] | NaCl | pignut hickory | dead | 0.01[b] | | |
| Direct[d] | NaCl | pignut hickory | ns | 0.01[b] | 0.42 | 0.12 |
| Direct[d] | $CaCl_2$ | American elm | st, y | 0.03[b] | | |
| Direct[d] | $CaCl_2$ | sugar maple | st, y | 0.03[b] | 0.10 | 0.11 |
| Direct[d] | $CaCl_2$ | black oak | ns | 0.03[b] | 0.04 | 0.03 |
| Run off, P[e] | NaCl | sugar maple | ns | 0.0 | 0.12 | 0.05 |
| Run off, D[e] | NaCl | sugar maple | ns | 0.0 | 0.05 | 0.05 |
| Run off, P[e] | $CaCl_2$ | sugar maple | ns | 0.0 | 0.29 | 0.07 |
| none | none | oak | ns | 0.0 | 0.02 | 0.06 |
| none | none | hickory | ns | 0.0 | 0.03 | 0.01 |
| none | none | maple | ns | 0.0 | 0.05 | 0.05 |

[a]ns = no symptoms; st = leaf stunting; y = leaf yellowing.
[b]Composite samples.
[c]Where no data are given, tree was removed before sampling.
[d]Weekly applications each at 50 lb/acre, made throughout the year directly over roots.
[e]Fifteen weekly applications, each at 15 lb/100 linear ft of road, made each winter on adjoining banked road; P = paved road; D = dirt road.

most of the Cl taken up by the deciduous trees was accumulated in their leaves and then removed from the trees when the leaves fell each autumn.

Eaton, who used hydroponic techniques, reported that Cl concentrations in his plants became 30–100 times greater than those in nutrient solutions (8). In the present experiments, leaf concentrations as great as 45 times the soil concentration were detected.

Experience with other leaf troubles, such as injury from insect feeding, leaf spot, blotch, anthracnose, damage from frost, and certain types of herbicide injury, suggests that if only the leaves are harmed, and especially if food reserves are not depleted by excessive loss of leaf area or by production of a new set of leaves the same season, the over-all health of the tree may not be seriously threatened. Therefore, differential accumulation of Cl in the foliage, rather than in the twigs, may protect the more permanent tissues of the tree from any damage that would result from high concentrations of Cl in those tissues.

An increase of Cl in twigs was detectable in trees salted weekly throughout the year. Thus, applications each winter to the run-off plots are being continued. Cumulative ill effects may have been too slight to be noticed at the end of only 7 winters of treatment (2, 12).

Strong (26) observed that red and white oak are far more tolerant of $CaCl_2$ than are American elm, black cherry, paper birch, hard maple, American beech, aspen, cottonwood, red and white pine, hemlock, white spruce, and balsam fir. Butijn (5) listed English oak among the trees most resistant to Cl. Harper (10) found bur oak among the least susceptible. Although Matukhin (20) found oak seedlings more sensitive to Cl than to sulfate, he considered oak capable of enduring a comparatively high soil salinity. In the present study, both white and black oak showed no symptoms or only slight foliar chlorosis when subjected to the same treatments that killed or severely injured maple, pine, birch, hickory, and elm.

The Cl contents detected in the treated oaks were lower, both for leaves and for twigs, than were the Cl contents of the other salted trees analyzed (Table 1). It seems possible, therefore, that the resistance of oak to injury from road salting is not the result of unusual Cl tolerance on the part of the oak tissues but rather the result of a tendency on the part of oak roots not to pick up the Cl, or both. Choice of rootstock has already been shown to influence the amount of Cl accumulated by avocado trees (7).

Oaks in some sites have tap roots that extend deeper than soil layers which might be affected most by salt applications. Larger pecan trees, with deeper root systems, however, have been affected more severely by salt in the soil than smaller pecans (10).

If salt was indeed taken up by the oak roots in the present experiment, most of it apparently did not reach the twigs or leaves (Fig. 1). Matukhin presents photographs showing considerable root injury to oak seedlings treated with NaCl (20). Such injury might prevent the roots from transmitting Cl to the rest of the tree, but the root injury itself would be expected to cause above-ground symptoms. If such injury occurred to the larger oaks in the present experiments, it was not severe enough to cause much foliar response.

NaCl has been more toxic to pine and elm (26) and

to avocado (1) than $CaCl_2$. In the present study, the number of trees to which $CaCl_2$ was applied directly throughout the year was too small for a definite conclusion. Nevertheless, symptoms on these trees did seem milder than those on trees in comparable NaCl plots.

Because the most severe symptoms of injury appeared in the leaves in which the measured Cl concentrations were greatest, it seems reasonable that injury was caused by the action of the Cl upon the plant tissues. Indeed, in some cases, injury has been caused by salt that was applied directly to the foliage, not to the soil or roots (27, 29). Moreover, specific Cl toxicity has been demonstrated by several investigators (1, 3, 4, 8, 12, 28). Severity of leaf injury to avocado has varied approximately as the Cl content of the leaves (1, 2, 7).

Nevertheless, the lower fertility associated with the presence of NaCl in the soil has been ascribed to altered physical properties of the soil and decreased availability of Ca (23). The injury caused by NaCl has been ascribed to the effects on soil characteristics of replacement of K by Na: a loss of organic matter, a dispersal of soil particles, a "puddling" of the soil, and a change from a base-exchange system to an acid-exchange system by which the soil becomes alkaline in weathering (22). Presumably during this period, K at first is abundant but later is deficient.

In the present experiments, the soil in the direct-application plots (salted throughout the year) did indeed become progressively less acid, changing from initial pHs of 4.1, 4.8, 5.0, and 5.9 to final pHs of 6.3, 6.5, 5.8, and 6.3, respectively. This change, however, brought the soil pH for most of the trees into the range considered most favorable for their growth. In the run-off plots, there was no equivalent trend, comparable initial pHs being 5.1, 5.4, and 6.5; and final pHs being 5.6, 5.6, and 5.7.

Moreover, after 200 weekly applications of salt, the treated soil appeared indistinguishable from nearby untreated soil, relative to organic matter in the top layer or texture in the various layers. K, however, was uniformly low (less than 60 ppm) in the 6 plots in July, 1958.

Matukhin's oak seedlings (20) were somewhat shorter and had a somewhat smaller root system when grown in soil treated with 0.2 *M* $Na_2SO_4$ (which gave a soil concentration of 1.06% or 10,600 ppm) than when grown in soil with no added salt, but they were far smaller in both respects when grown in soil treated with 0.2 *M* NaCl (which gave a soil concentration of 0.44% or 4,400 ppm). Matukhin's figures were based on "completely dry weight" of soil, presumably oven-dried soil.

Apparently, the Na in Matukhin's treatments had a deleterious effect but the Cl had a much more severe effect. Na injury to avocado foliage does not resemble Cl injury (1, 2). Na does not accumulate in peach foliage, as Cl does, although it does accumulate in peach twigs and roots (12).

Osmotic effects also have been implicated (3, 13, 18). Several mechanisms may operate at once to produce salt injury to plants (3, 11).

Plice (22) found improvement in salted soil over a period within which 25 in. of rain fell. Precipitation for the entire period of the present experiments (from December, 1954 through July, 1958) was 156 in., an average of 45 in./year.

Eaton (8) found that tomatoes were stimulated by 3 meq of Cl (106.5 ppm) in nutrient solutions. In the present experiments, no injury was detected when salt concentrations in the soil were so low.

Using rooted lemon cuttings in nutrient solutions, Eaton found reduced growth at 50 meq of Cl (1,773 ppm) and death at 150 meq (5,319 ppm). In the present experiment, death of maples occurred when soil soluble salts were no more than 22.5 meq (800 ppm), although leaf Cl concentration reached as high as 1,025 meq (36,400 ppm), based on air-dry weight.

Eaton reported no leaf symptoms but merely a depressed growth. The present experiments yielded striking leaf symptoms.

Eaton also found that the degree of injury to his lemon cuttings could be correlated with the amount of salt accumulated in the sap. The present leaf and twig Cl analyses tend to confirm this result. Thus, regardless of whether the mechanism of injury lies in soil changes or in Cl effects on tissues, a knowledge of the Cl content of the living tissues of trees injured by salt applications will be useful in future diagnoses. Differences in salt tolerance, however, may be shown not only by different tree species (26, 29, 31), but also by individual trees of the same species (28). Moreover, the decreased Cl content in maple twigs a year after death of the tree suggests that diagnosis of salt injury should not be based on analysis of dead tissues.

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# HOST RANGE, SPORE GERMINATION, AND PATHOGENICITY OF DIPOROTHECA RHIZOPHILA

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## SUMMARY

Fifty-four of 56 *Solanum* spp. proved susceptible to *Diporotheca rhizophila* Gordon & Shaw, a root-parasitizing species of the Meliolaceae (black mildews). Species belonging to other genera of higher plants were not found infected. Potatoes (*S. tuberosum* L.) and eggplant (*S. melongena* L.) were the only economically important species found susceptible. Of the 3 varieties of potatoes tested, Russet Burbank was susceptible; White Rose, resistant; and Pontiac, apparently immune. Inoculation was accomplished by inserting a glass plate coated with ascospores against 1 side of several boxes in which eggplant and nightshade were planted in sterilized soil. Infection occurred only on those roots that, in elongating, contacted the ascospores.

In germination, either or both of the 2 small

polar cells of the 3-celled ascospore produce germ tubes terminating in characteristic 2-celled hyphopodia. An infection peg from the hyphopodium penetrates the host cell, forming either a stylet-type, or more rarely, a bulbous haustorium. Spore germination and infection were obtained with ascospores collected from naturally infested soil by a flotation process. Fresh ascospores, or ascospores wintered for only 1 season, did not germinate. Morphologically, *D. rhizophila* is considered an obligate, but weakly pathogenic parasite. The fungus survived for at least 5 years in infested soil stored in the greenhouse under a variety of conditions. Ascospores, the only type of spore produced, are considered the structures surviving in the soil and serving as inoculum.

INTRODUCTION.—*Diporotheca rhizophila* Gordon & Shaw (4), a member of the Meliolaceae, forms a dense superficial reticulum of dark brown mycelium on the fibrous root systems of *Solanum* spp. It is the only known subterranean member of this family (6) which typically comprises tropical leaf parasites, the black mildews. *D. rhizophila* has occurred on potatoes (*S. tuberosum* L.) and eggplant (*S. melongena* L.) in the same experimental plots at the Irrigation Experiment Station, Prosser, Wash., each year since it was first observed in 1944 (5, 11). Furthermore, it has consistently developed in the greenhouse whenever these species have been planted in soil taken from the infested plots. The ease with which infection was obtained led to a study of the host range of the fungus, artificial inoculation techniques, observations on ascosporic germination, spore longevity, and the establishment of the host-parasite relation.

HOST RANGE STUDIES.—*Materials and methods.*—Forty-three tuber-forming *Solanum* spp. were obtained from the Plant Introduction Station at Sturgeon Bay, Wis. Of the 3 or more tubers received of each species, 2 were planted directly in the experimental plots at Prosser and the rest in naturally infested soil in the greenhouse at Pullman. In addition, the following commercial cultivated potato varieties were tested more extensively: Pontiac (red-skinned), White Rose (white-skinned), and Russet Burbank (netted skin; commonly called the Netted Gem). Twelve *Solanum* spp. (all nontuber-forming), obtained from various sources in the United States and Mexico, also were tested.

Eighteen species and varieties representing 10 other genera of the Solanaceae were tested in the greenhouse and in the field. They included: *Atropa belladonna* L., *Capsicum frutescens* L. var. *grossum* (D.C.) Bailey, *C. frutescens* L. var. *longum* Bailey, *Datura stramonium* L., *Hyoscyamus niger* L., *Lycopersicon esculentum* Mill., *L. glabrum* Mull., *L. hirsutum* H.B. K., *L. peruvianum* Mill., *Nicandra physalodes* (L.) Gaertn., *Nicotiana attenuata* L., *N. glutinosa* L., *N. tabacum* L., *Petunia hybrida* Vilm., *Physalis floridana* Rydb., *P. heterophylla* Nees, *P. peruviana* L., and *Schizanthus hybrida* Hort.

The following nonsolanaceous species were tested in the field only: corn (*Zea mays* L., Gramineae); alfalfa (*Medicago sativa* L., Leguminosae); yellow sweet clover (*Melilotus officinalis* Lam., Leguminosae); beans (*Phaseolus vulgaris* L., Leguminosae); and sugar beet (*Beta vulgaris* L., Chenopodiaceae). All of these agronomic species are cultivated extensively in the Columbia River Basin. In addition, the roots of other cultivated species and of indigenous and established weed species growing on known infested soil were checked repeatedly for evidence of infection.

*Results.*—The presence on the fibrous roots of dark brown mycelium bearing capitate hyphopodia is diagnostic and easily ascertained (4). Fifty-four of the 56 *Solanum* spp. tested were susceptible; only *S. elaeagnifolium* Cav. and *S. triflorum* Nutt. remained uninfected. In these inoculation tests, the susceptibility of *Solanum* spp. to *D. rhizophila* on a scale of 0 – 4 (0 = immune, no mycelium present; 1 = resistant, very small mycelial colonies; 2 = susceptible, with large colonies on roots, no apparent necrosis; 3 = susceptible, with roots colonized extensively, slight necrosis; 4 = extreme susceptibility, roots completely colonized, extensive epidermal necrosis) was: 0 — *S. elaeagnifolium* Cav., *S. triflorum* Nutt., and *S. tuberosum* L. var. Pontiac (Hort.); 1 — *S. tuberosum* L. var. White Rose (Hort.); 2 — *S. acaule* Bitter, *S. acroscopicum* Ochoa, *S. ahanhuiri* Juz. & Buk., *S. alandiae* Cárd., *S. berthaultii* Hawkes, *S. bulbocastanum* Dun., *S. canasense* Hawkes, *S. capsicibaccatum* Cárd., *S. cardiophyllum* Lindl., *S. chacöense* Bitter, *S. chiquidenum* Ochoa, *S. ciliolatum* Mart. & Gal., *S. commersonii* Dun., *S. curtilobum* Juz. & Buk., *S. dulcamara* L., *S. ehrenbergii* Rydb., *S. famatinae* Bitter & Wittm., *S. gandarillasii* Cárd., *S. goniocalyx* Juz. & Buk., *S. gilo* Raddi, *S. indicum* L., *S. lignicaule* Vargas, *S. maglia* Schlecht., *S. mammosum* L., *S. neohawkesii* Vargas, *S. pampasense* Hawkes, *S. phureja* Juz. & Buk., *S. pinnatisectum* Dun., *S. piurae* Bitter, *S. polyadenium* Greenm., *S. polytrichon* Rydb., *S. quitöense* Lam., *S. rostratum* Dun., *S. sambucinum* Rydb., *S. sanctae-rosae* Hawkes, *S. setulosistylum* Bitter, *S. simplicifolium* Bitter, *S. sodomeum* L., *S. sogarandinum* Ochoa, *S. soukupii* Hawkes, *S. sparisipilum* (Bitter) Juz. & Buk., *S. spectabile* (Correll) Hawkes, *S. stenotomum* Juz. & Buk., *S. stoloniferum* Schlecht. & Bouche, *S. sucrense* Hawkes, *S. tarijense* Hawkes, *S. toralapanum* Cárd. & Hawkes, *S. vernei* Bitter & Wittm., *S. verrucosum* Schlecht., *S. xerophyllum* Hawkes; 3 — *S. melongena* L., *S. tuberosum* L. var. Russet Burbank (Hort.); 4 — *S. demissum* Lindl., *S. nigrum* L.

Nightshade (*S. nigrum*) and *S. demissum* were the most heavily infected. *S. tuberosum* and *S. melongena* were the only susceptible species of economic value. The Russet Burbank potato variety was susceptible; the White Rose variety displayed resistance; and Pontiac appeared immune. *Solanum spp.* that became in-

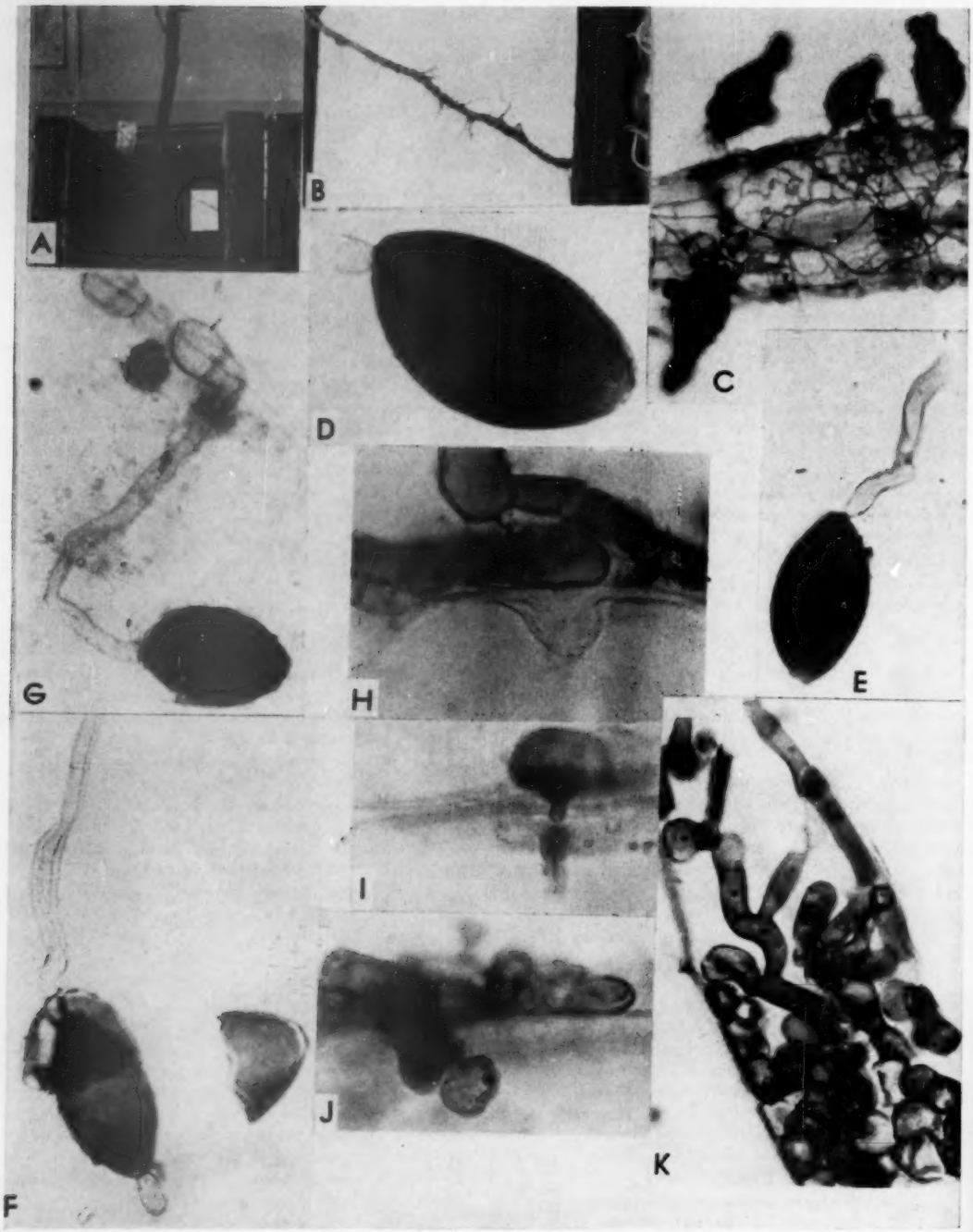


**Fig. 1.** *Diporotheca rhizophila.* **A)** Inoculation box showing infected root of *Solanum nigrum* L. (× 0.25). **B)** Infected root (× 2). **C)** Infected root enlarged to show superficial mycelium and perithecia (× 510). **D)** Initial stage in germination of the ascospore (× 1,400). **E)** Germinating ascopore showing 1 germ tube (× 830). **F)** Germinating ascospore producing 2 germ tubes; note fractured sheath (× 875). **G)** Germinating ascospore, germ tube, and initial hyphopodia (× 700). **H)** Development of penetration peg from terminal cell of hyphopodium causing thickening and invagination of host epidermal cell wall (× 930). **I)** Stylet-type haustorium (× 930). **J)** Nucleus of host cell adjacent to the flattened haustorium (× 930). **K)** Mycelium and hyphopodia (× 1,050).

fected in the field also were infected in the greenhouse; field and greenhouse susceptibility readings agreed closely. A few species (*e.g., S. piurae*) failed to grow in the field. Hence, although susceptible in the greenhouse, they could not be proven susceptible under field conditions. No solanaceous species, other than *Solanum* spp., and no nonsolanaceous species growing in known infested soil were attacked by this fungus.

INOCULATIONS.—*Methods.*—Eggplant and nightshade were used in greenhouse trials. Seeds were germinated in an autoclaved 1:1 mixture of sand and peat moss. When the seedlings were 10–12 days old, they were transplanted into sterilized soil from the Prosser plots in cedar boxes measuring 6 x 6 x 12 in. high. One side of each box was hinged. Ascospores and perithecia were obtained from unsterilized, infested plot soil by flotation (10). The detergent Tween 20 (polyoxyethylene sorbitan monolaurate; Atlas Powder Co., Wilmington, Del.) was used in place of mineral oil. An ascosporic suspension, with Tween 20 added as a spreader-sticker, was applied to glass plates measuring 4 x 9 in. One of these glass plates was inserted against the hinged door in each of 2 boxes, with the side of the glass plate bearing the spore suspension in contact with the soil. A glass plate without a spore suspension was inserted in 1 additional box. Four eggplant seedlings were transplanted into each of the 3 boxes, 2 inoculated and 1 control. This arrangement was duplicated with nightshade. After the transplants were well established, the number of plants/box was reduced to 2.

*Results.*—Final observations were made 100 days after transplanting. By this time, the roots were thickly crowded against the glass plates. In each of the inoculated boxes, several colonies of *D. rhizophila* were found on the roots of both eggplant and nightshade (Fig. 1-A, B, C) where they had come in contact with the inoculum on the glass plates. These colonies were producing perithecia (Fig. 1-C), containing mature ascospores. No colonies were found on roots not in contact with the inoculated glass plates, nor did any colonies develop in the uninoculated controls.

SPORE GERMINATION.—*Materials and Methods.*—Numerous single ascospores obtained from fresh perithecia and from perithecia overwintered 1 season outdoors were placed on many types of media, including media augmented with expressed root sap of potatoes, eggplant, and nightshade. Germination trials were conducted at various constant temperatures from 5–35°C. In other trials, the temperature was varied between these extremes.

Aseptic eggplants growing on Bonner's modified pea medium (14) were inoculated with single ascospores from fresh perithecia. These inoculated seedlings were maintained at laboratory temperatures (20–24°C) for 24 months.

Field observations and greenhouse plantings in infested soil had shown that infection occurs, and mature perithecia are produced within 35 days. Therefore, 60 Russet Burbank potato plants were started from seed pieces in naturally infested soil in the greenhouse. After 20 days, 3 plants were removed each day, washed carefully in water, and observed under a dissecting microscope. Nightshade and eggplant also were employed in similar tests: the seeds were germinated in sterilized soil and the seedlings transplanted after 10 days to infested soil. On the 15th day after transplanting and each day thereafter, 3 seedlings of each species were removed and examined.

*Results.*—All laboratory attempts to induce germination of fresh ascospores and of ascospores overwintered 1 season failed.

Colonies of the fungus could not be differentiated on the fibrous root systems of potatoes grown in infested soil prior to the 22nd–24th day after planting because of the numerous root hairs to which soil and humus particles cling. By this time, the colonies were already 2–3 mm in diameter, and mycelial development was so profuse that it was impossible to locate germinating ascospores. Similar difficulties were encountered with eggplant. Nightshade, however, produces white, fibrous roots bearing few root hairs. Germinating ascospores were first observed on the roots of this species on seedlings removed and washed the 18th day after transplanting into infested soil. Two–several germinating ascospores were found on the roots of every seedling examined on the 19th and 20th days. Therefore, the majority of the seedlings were removed, and either fixed for sectioning, or put in cold storage for subsequent study.

At the time of germination, the sheath around the ascospore usually is still intact and has a purple sheen. At a magnification of 45 ×, ascospores are easily seen against the white roots of nightshade. The small apical cells of the ascospores germinate, the germ tube emerging through the apical pores in the sheath (Fig. 1-D, E). The sheath frequently fractures during germination. We have found no indication that the large central cell is functional, although modifications occur in its contents (Fig. 1-D). Two germ tubes (Fig. 1-F), 1 from each of the 2 apical cells, normally are produced. In the majority of the germinating spores, 1 apical cell produces a germ tube 3–4 days before the other (Fig. 1-E). The germ tube has been traced several times to the initial hyphopodium (Fig. 1-G) which is formed 50–150$\mu$ from the ascospore. The germ tube and the 1st hyphopodium are initially light tan, becoming progressively darker.

Care must be exercised in dissecting the portion of the root bearing a germinating ascospore and in transferring it to a temporary or permanent mount. Excessive pressure easily detaches the ascospore from the germ tube. This, together with the excessive washing necessary to remove soil debris from the roots of potatoes and eggplant, probably explains our inability to find ascospores in association with young colonies of the fungus on seedlings of these 2 hosts.

SPORE LONGEVITY IN THE SOIL.—Soil naturally infested with *D. rhizophila* was kept in the greenhouse in containers for 5 years. The containers were maintained under different conditions (air dry, soil fallow

but moist, cropped continually to oats, and cropped continually to eggplants). *D. rhizophila* developed on the roots of eggplants planted in this soil after 5 years, regardless of the conditions under which the soil was maintained during the interval.

In a field of 10 acres at Satus, Yakima County, Wash., in which potatoes had not been planted during the previous 12 years, 100% infection by *D. rhizophila* occurred on potatoes in 1959. Nightshade (*S. nigrum*), an occasional weed in the field, also was infected.

A similar situation was found in a field of potatoes in the Badger Pocket area (Kittitas County). The fungus was most profuse on both potatoes and nightshade in a poorly drained corner of the field. The owner stated that a fairly heavy infestation of nightshade had been present there for several years. Potatoes growing elsewhere in the field, though infected, were not so heavily parasitized. According to the owner's records, this field had been used primarily for pasture and hay crops and had not been planted to potatoes or any other commercial *Solanum* spp. since 1949.

Host-Parasite Relations.—The observations presented here were made in a detailed study of the morphology of *D. rhizophila* (4). The dark brown superficial mycelium consists of uninucleate, cylindrical cells bearing 2- or 3-celled, capitate hyphopodia (Fig. 1-K). The terminal cell of each hyphopodium is always the larger (or largest). When a hyphopodium is observed from above, a minute circular ring is often discernible within the terminal cell. This ring is actually a thickening of the lower hyphopodial wall where the penetration peg is formed (Fig. 1-H, I). The development of the penetration pegs is easily followed in radial and transverse sections of infected roots. A single small protrusion from the underside of the terminal cell pushes against the epidermal cell wall (Fig 1-H), which becomes thickened and indented. Frequently, the penetration peg itself functions as a haustorium and might be termed a stylet-type haustorium (Fig. 1-I). As many as 6 stylet-type haustoria have been observed in a single host cell, although 2 or 3 are common. In relatively rare instances, the tip of the penetration peg enlarges to form a small bulbous haustorium (Fig. 1-J). These bulbous haustoria occurred singly within epidermal cells. The nucleus of the terminal cell of the hyphopodium migrates into the haustorium without division; thus, the terminal cell of the hyphopodium is anucleate when the haustorium is fully developed. In several instances, the nucleus of the host cell was adjacent to the haustorium, the haustorium being flattened or indented (Fig. 1-J). Hawker (9) cites several examples among other obligate parasites (*e. g., Puccinia graminis* Pers.) of migration of the host cell nucleus toward the invading haustorium.

Collapse and death of individual host epidermal cells occur in areas of extensive hyphopodial and haustorial development. Subsequently, hyphae invade the lumina of the dead epidermal cells and produce additional hyphopodia which send haustoria into the underlying cortex. Cells of this layer of the cortex in turn may collapse and be invaded by hyphae. Penetration pegs and haustoria have been observed in the 1st and 2nd layers of cortical cells, but not in deeper layers.

At the time of harvest, the cortex of potato roots heavily infected with *D. rhizophila* readily sloughs away from the central stele. Nevertheless, no aboveground symptoms have been associated with heavy infections of the roots by *D. rhizophila*. Nor have the tubers been found infected; thus, the size and number of tubers collected from infected plants are apparently not reduced.

Discussion.—On the basis of its morphology and our failure to culture it on artificial media, *D. rhizophila* is considered an obligate parasite like other members of the Meliolaceae (13). Observations of the degree of its pathogenicity, however, are as yet inconclusive, since this fungus has not been successfully inoculated on any of its hosts in the absence of other organisms. On potatoes particularly, mycelium of *Rhizoctonia solani* Kühn also is commonly present. Consequently, on this host, the death and necrosis of epidermal and outer cortical cells cannot yet be attributed solely to *D. rhizophila*. *R. solani* occurs much less frequently on *S. nigrum*. Collapse and death of epidermal cells have been observed on this host in association with the mycelium of *D. rhizophila* in the absence of other fungal mycelia.

Apparently, *D. rhizophila* is at most weakly pathogenic. This agrees with Stevens' (12) statement that the majority of the species of the Meliolineae (*i.e.*, Meliolaceae with phaeophragmous spores) cause no visible effects on their hosts.

Some 2,000 species have been described in the Meliolaceae. It has become an accepted hypothesis in taxonomic studies of the group (2, 7, 8,) that each species is restricted in its host range to a single host genus. The "strict" or obligate parasitism (13) of these fungi, and hence their similarity to species of the Uredinales, Erysiphales, and Peronosporaceae, has no doubt contributed to this concept. Previous attempts at artificial inoculation to determine host ranges for species of the Meliolaceae have been unsuccessful or inconclusive (2). Toro (13) states: "There is no record of the time the infection takes place or of the conditions of the host necessary for infection" in any species of the Meliolaceae. Hansford (personal communication) states that no worker has ever germinated a spore of any species of the Meliolaceae on artificial media nor previously inoculated successfully a susceptible host plant with a species of this family.

*Solanum* spp. included in these inoculation trials constitute only a small fraction of the approximately 1,500 species which have been described (1). Yet, it is interesting to note that of the 56 species tested, only 2 (*i.e.*, less than 4%) failed to become infected. Conversely, the failure to obtain infection on any of the species belonging to other genera of the Solanaceae and on nonsolanaceous species suggests that parasitism of *D. rhizophila* is limited to *Solanum* spp.

These inoculation studies support the hypothesis that each species of the Meliolaceae is restricted to a comparatively narrow range of hosts. While subsequent inoculation experiments may demonstrate that not all species of the Meliolaceae are limited to a single host genus, these results indicate such host specialization for *D. rhizophila*. The use of the systematic position of the host plant as a character in the definition of fungal species in the Meliolaceae and in other groups of obligate parasites must initially be considered provisional. Mounting evidence suggests, however, that this criterion is not entirely artificial. The evidence presented here of the usefulness of the host substrate in species delimitation in yet another group of obligate parasites strengthens the species concepts proposed by Fischer and Shaw (3) and Yerkes and Shaw (15) for such fungi.

The apparent existence of *Solanum* spp. which are immune to *D. rhizophila*, and the demonstration of variable susceptibility among the commercial varieties of *S. tuberosum* parallels our knowledge in regard to many other obligately parasitic fungi.

The artificial inoculations reported herein and the direct observations of ascosporic germination prove that the ascospores serve as inoculum of this fungus. Since the fungus is an obligate parasite, it is improbable that the mycelium persists for any period of time in the absence of living roots of susceptible hosts. The absence of other spore stages or vegetative structures which might function as disseminules suggests that ascospores constitute the only inoculum produced by this fungus.

The failure to obtain germination of freshly produced ascospores and of those overwintered 1 season, even when these were placed in contact with the roots of susceptible species, indicates that the ascospores must be exposed to weathering (or aging) for more than 1 season. At present, it can only be stated that germinable ascospores can be demonstrated in the soil of the Prosser experimental plot at all times of the year by infection tests.

It has been demonstrated that *D. rhizophila* can persist in soil for at least 5 years even though no susceptible host is grown in the soil during that period. The results of this experiment suggest that the occurrence of the fungus on potatoes at Satus, Wash., after a 12-year interval between potato crops, may have been due to survival of the fungus (as ascospores) in the soil, although perpetuation on nightshade (*S. nigrum*) or new introductions cannot be ignored entirely.

Hansford (personal communication) states that many species of the Meliolaceae are known only from single collections and are apparently of limited geographical distribution. Other species are widely distributed, particularly in the tropics, and occur wherever their host plants grow. Hansford also indicates that generally the Meliolineae parasitize only the indigenous plants of any particular area, though in some instances they may spread from these to introduced or cultivated plants closely allied to their native hosts.

In contrast to this general rule, it is hypothesized that *D. rhizophila* was introduced into Washington together with one of the hosts (probably potatoes) susceptible to it. The tropical habitat of most species of both the Meliolaceae and the genus *Solanum* suggests that the fungus is indigenous to equatorial regions. Failure to find the fungus anywhere in Washington or the Pacific Northwest, except in the Yakima River Valley (4), and the immunity of the only *Solanum* sp. native to Washington (*S. triflorum* Nutt.) also suggest that the fungus has been introduced. The fungus may be indigenous to Europe from whence both the nightshades (*S. nigrum L.* and *S. dulcamara* L.) were introduced.

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# THE SPREADING DECLINE COMPLEX: THE SEPARATE AND COMBINED EFFECTS OF FUSARIUM SPP. AND RADOPHOLUS SIMILIS ON THE GROWTH OF DUNCAN GRAPEFRUIT SEEDLINGS IN THE GREENHOUSE

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SUMMARY

*Radopholus similis* and *Fusarium* spp. together caused more severe root damage in Duncan grapefruit seedlings grown under greenhouse conditions than either *Radopholus* or *Fusarium* spp. alone.

INTRODUCTION.—Spreading decline, a serious root disorder of citrus trees in Florida, was named and described by Suit in 1947 (10). In the course of their work, Suit and DuCharme evaluated the possible roles of various organisms in the disease and concluded that there were no differences in the microflora of decline areas compared with healthy areas and that viruses were not involved. In 1947 (10), *Tylenchulus semipenetrans*, and in 1949 (11) and 1950 (12) *Fusarium* sp. were suspected as the disease organism. In 1953 (9), Sherbakoff reported that the same fungi, in different proportions, were found in various decline-affected and decline-free areas and concluded that *Fusarium* has no direct pathogenic relation to the disease. In the same year, Suit and DuCharme (13) found the burrowing nematode, *Radopholus similis* (Cobb) Thorne, associated with the feeder roots of declining trees and subsequently stated that this nematode is the primary cause of the disease. In 1953, Suit, *et al.* (14) described the possible progression of pathogenic fungi, as secondary invaders, into feeder roots following invasion by *Radopholus*. They reported finding *Phytophthora*, *Pythium*, *Fusarium*, *Penicillium*, and other forms in both healthy and spreading-decline trees. *Fusarium* and *Penicillium* were most frequently encountered. Suit, *et al.* also succeeded in producing symptoms of the disease under experimental conditions using *Radopholus*-infested subsoil and considered their findings as strong evidence that *Radopholus* is the causal agent of spreading decline. In 1954, DuCharme (2), in a review of the research on spreading decline, considered the possible involvement of various factors such as fungi, actinomycetes, bacteria, viruses, soil toxins, and nematodes. He cited evidence that none of these except nematodes was the cause; and showed how this work led to the decision that *Radopholus* was the cause of spreading decline.

With the possible exception of work by Suit, *et al.* (14), only single factors were evaluated for their possible involvement as causative organisms of the disease. Other reports since 1953 implicate *Fusarium* with the disease. Feder, Feldmesser, and Walkinshaw (4) were able to recover *Fusarium* sp. from xylem of the crowns of Duncan grapefruit seedlings infected with the burrowing nematode. Feldmesser, *et al.* (5) demonstrated that a build-up of *Fusarium* followed the migration of burrowing nematodes from a single naturally infected citrus seedling through steam-sterilized soil into citrus roots previously free of nematodes. They also found that burrowing nematode-infected roots always contained *Fusarium* spp. in greater proportions than nematode-free roots.

Pertinent observations were made by the authors on Duncan grapefruit seedlings grown in burrowing nematode-infested subsoil taken from beneath a citrus tree and an avocado tree growing adjacent to one another in a grove. The roots of both sets of seedlings contained an average of 145 *R. similis*/g of root but only those seedlings growing in subsoil from beneath the citrus tree showed decline symptoms.

Duncan grapefruit seedlings were planted into steam-sterilized and untreated subsoil from beneath the same avocado and citrus trees. The seedlings planted in untreated avocado subsoil or steamed citrus or steamed avocado subsoil were vigorous and showed no decline symptoms, whereas those planted into untreated citrus soil showed typical decline symptoms. *R. similis* was recovered from the roots of seedlings planted into untreated avocado and citrus soils but not from those planted into steamed soils. Thus, no relationship appeared between the presence of *R. similis* and spreading decline in the avocado subsoil.

These data indicated that spreading decline may be due to a complex of disease organisms.

MATERIALS AND METHODS.—Two separate, essentially similar, experiments were done. Duncan grapefruit seedlings were germinated from peeled, surface-sterilized seed, and grown in steam-sterilized sand. Peeled seeds were soaked 20 minutes in calcium hypochlorite solution (5% active chlorine) and planted in steam-sterilized sand.

Burrowing nematodes were reared aseptically on excised okra root tip tissue by the following method. Okra seed was surface-sterilized in calcium hypochlorite (5% active chlorine) for 20 minutes and germinated in the dark at 28°C on sterile filter paper in petri dishes. Root tips 10–30 mm long were excised aseptically and placed on semisolid (1% agar) White's modified medium (16) in 200-ml medicine bottles. Two weeks later, adult females of *R. similis* were surface-sterilized as described by Feder (3), removed from the centrifuge tubes with a bit of sterile cellulose sponge, and placed on the agar next to the growing okra roots. Inoculated cultures were incubated for 3

months at 27°C and nematodes were recovered from the cultures by incubation in sterile, distilled water as described by Young (17). Fifty nematodes were used to inoculate those pots designated to receive nematode inoculum. A hole was made next to the root system with a sterilized glass rod and 50 nematodes were absorbed on a piece of sterile cellulose sponge which was placed in the hole and covered with soil.

Fungal inoculations were made in 2 ways. When fungi were added at the start of the experiment, roots of seedlings were dipped in suspensions of fungal mycelium and spores and then planted in sterilized soil. When the fungi were added to established, growing seedlings, fungal suspensions were poured into aseptically made holes in the soil around the root systems.

Fungal inoculum for the first experiment was a homogenate of hot-water-treated, blenderized burrowing nematode-infected Duncan grapefruit seedling feeder roots. Such nematode-infected feeder roots, when freed of their nematodes by a 10-minute soak in water at 122°F as described by Birchfield (1), still retained a viable fungal population. Since there was no sure way of knowing which fungus might be involved in a possible complex, whole nematode-free root homogenate was used.

For the second experiment, *Fusarium oxysporum* Schlecht. and *F. solani* (Mart.) App. Wr. were used. These were isolated from the crowns of Duncan grapefruit seedlings at the conclusion of the first experiment.

EXPERIMENT 1.—Four treatments and a check were used. There were ten 6-in. pots/treatment and 2 plants /pot. Plants were inoculated with fungi only (heated root homogenate), with burrowing nematode only, with fungi followed 1 month later by burrowing nematodes, and with burrowing nematodes followed 1 month later by fungi. The check plants were planted without treatment.

EXPERIMENT 2.—Three treatments and a check were used. There were ten 6-in. pots/treatment and 1 plant /pot. Plants were inoculated with fungi only (2 strains of *F. oxysporum* and 1 of *F. solani* isolated from crowns in experiment 1), with burrowing nematodes only, and with burrowing nematodes followed in 1 month by *Fusarium* spp. Controls were planted with no treatment.

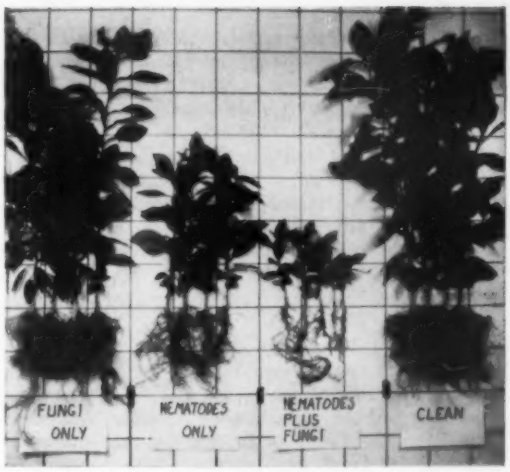


**Fig. 1.** Duncan grapefruit seedlings 10 months after inoculation with fungi only (*Fusarium*), nematodes only (*Radopholus*), and nematodes + fungi (*Radopholus* + *Fusarium*), compared with uninoculated check seedlings.

RESULTS.—Experiment 1 was run for 18 months. Plants in the fungus treatments and controls were pot-bound and the incidence of *Fusarium* spp. recovery from the crowns of all treatments was higher, probably due to contamination from the water and air. In the case of seedlings inoculated with *R. similis* but no fungus at the start of the experiment, there was a gradual increase in the percentage of infected crown pieces. At the end of the experiment, the percentages

TABLE 1. Summary of results of 2 experiments in which roots of Duncan grapefruit seedlings were inoculated with populations of the burrowing nematode, *Fusarium* spp., and combinations of these 2 pathogens

| Treatment | Total length (cm) | Crown diam. (mm) | Dry wt.[a] roots (g) | % crown pieces with *Fusarium* (6 pieces/plant) | *R. similis*/g roots |
|---|---|---|---|---|---|
| | | *EXPERIMENT 1* | | | |
| *Fusarium* | 92.1[b] | 8.9 | 14.8 | 38.0 | 0.0 |
| *Radopholus* | 71.4 | 7.9 | 10.0 | 62.0 | 2.4 |
| *Fusarium* + *Radopholus* | 70.3 | 7.8 | 10.6 | 52.0 | 4.4 |
| *Radopholus* + *Fusarium* | 61.6 | 8.3 | 11.3 | 78.0 | 9.6 |
| Control | 94.5 | 9.7 | 17.9 | 7.0 | 0.0 |
| | | *EXPERIMENT 2*[c] | | | |
| *Fusarium*[d] | 68.4[e] | 7.6 | 4.2 | 12.0 | 0.0 |
| *Radopholus* | 44.9 | 5.4 | 2.1 | 7.0 | 52.7 |
| *Radopholus* + *Fusarium* | 28.5 | 3.4 | 1.0 | 53.0 | 48.0 |
| Control | 75.8 | 8.2 | 4.5 | 3.0 | 0.0 |

[a] Dry weight of roots after 48 hours at 60°C in a drying oven.
[b] Each figure represents the average of 20 plants/treatment.
[c] All differences are significant at the 1% level.
[d] *F. oxysporum* and *F. solani*.
[e] Each figure represents the average of 10 plants/treatment.

of infected crown pieces were in the same range as found in the fungus-nematode combinations. In spite of these difficulties, the plants inoculated with both nematodes and fungi were affected more severely than plants inoculated with either pathogen alone. The plants inoculated with fungi only did not show significant damage in roots or tops. No significant differences in crown diameter or dry root weight existed among groups of plants treated with nematodes followed by fungi, fungi followed by nematodes, or nematodes alone. Total plant growth, however, of those plants inoculated with nematodes and then fungi, was considerably less than in those plants treated with nematodes alone (Table 1).

Experiment 2 was carried out using only 1 plant/pot and was run 10 months. These slight revisions corrected the difficulties experienced in experiment 1 and increased the reliability of the data obtained in experiment 2. Effect of inoculating with nematodes followed by fungi was far more severe than when nematodes alone were used (Table 1). Total plant length, crown diameter, and dry root weight were affected more adversely by the former treatment than by the latter, and both treatments affected plant growth more severely than the addition of fungi alone (Fig. 1).

DISCUSSION.—The data indicate that combined infections of *Radopholus similis* and *Fusarium* spp. caused severe root damage in Duncan grapefruit seedlings under greenhouse conditions. Although *Fusarium* spp. can be recovered, generally, from burrowing nematode-infected roots, it seems more significant that *Fusarium* was recovered from the crowns of burrowing nematode-infected plants, especially from plants inoculated with sterilized nematodes followed, after a 1-month interval, by *Fusarium*. It seems clear, also, that the percentage of recovery of *Fusarium* from crowns was always enhanced by the presence of burrowing nematodes in the roots, as reported by Feldmesser, *et al.* (5). This occurred whether the fungus was inoculated into the plant roots or occurred as an air contaminant. It should be noted also that this is the first instance in which nematodes grown aseptically in tissue culture have been used to demonstrate a phytopathogenic fungus-nematode complex.

This information indicates that the etiology of spreading decline of citrus is similar to that involving root-knot nematodes and *Fusarium* spp. reported from such other crops as tobacco by Holdeman (6) and Morgan (7); cowpea by Thomason, Erwin, and Garber (15); and banana by Newhall (8).

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# PROTECTION OF CUCUMBER SEEDLINGS FROM STONE FRUIT RINGSPOT VIRUS BY 8-AZAGUANINE

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SUMMARY

Cucumber seedlings were protected from infection by stone fruit ringspot (RSV) by treatment with 8-azaguanine (AZA). The degree of protection, indicated by the inhibition of symptoms, varied directly with the AZA concentration, and inversely with inoculum titer. A preinoculation treatment plus a postinoculation treatment gave the best protection. In plants not completely protected, AZA delayed the appearance of systemic virus symptoms and reduced the severity of symptoms. AZA was more effective in suppressing local starch lesions than systemic virus symptoms; when incorporated in the inoculum, AZA, however, had no effect on starch lesions. A single preinoculation treatment caused 50% reduction in RSV starch lesions at 0.1 *mM* compared with a concentration of 0.7 *mM* for a 50% reduction in tobacco mosaic virus starch lesions. In addition to inhibiting primary virus replication sites in the cotyledons, AZA had a systemic effect and inhibited virus development in secondary leaves. Guanine was only partially effective in reversing either of these effects of AZA.

INTRODUCTION.—Of the various materials found to be effective for inhibition of virus replication (1, 5), 8-azaguanine (AZA) had the greatest latitude between the concentration required for virus inhibition and phytotoxicity. In studies on the mechanism of action of AZA, the material has been found to block a high percentage of tobacco mosaic virus replication sites and to inhibit virus translocation (2). While AZA was not effective in freeing plants of virus, it was reported effective in preventing infection of certain plants by alfalfa mosaic or cucumber mosaic viruses (6, 7). The present report concerns the effect of AZA on an unstable virus, stone fruit ringspot (RSV).

MATERIALS AND METHODS.—A stock culture of RSV was maintained in cucumber (*Cucumis sativus L.* "Chicago Pickling") by transfer at 1- or 2-week intervals. Inoculations were made by rubbing cotyledons twice with a standard cheesecloth pad saturated with inoculum containing 5% by weight of Carborundum. Inoculum was prepared by homogenizing cotyledons of 7- to 14-day infected plants with buffer (3) at the rate of 1 part by weight of tissue to 10 parts of buffer. Larger proportions of buffer were used for inoculum dilution studies. Buffer was composed of 0.007 *M* neutral phosphate, 0.01 *M* sodium diethyl barbiturate, 0.007 *M* neutral potassium ethylene diamine tetraacetate, and 0.01 *M* cysteine-HCl. Cucumber plants kept longer than 2-3 weeks were transferred to larger pots to support growth.

Plants were treated by vacuum infiltration (1) with AZA dissolved in 1-*mM* KOH. Starch lesions were counted under a low-power microscope (×12.5) after cotyledons had been cleared in alcohol and stained with IKI (4).

Cucumber does not develop a recovery phase after infection with RSV, and virus can be recovered only from tissue expressing symptoms. Since virus replication parallels symptom formation, the percentage of plants expressing symptoms gives a rough indication of virus titer. Similarly, the number of starch lesions is a measure of titer; however, it is possible that a small amount of virus may be present even though no starch lesions are evident. Both of these methods were used in measuring the effect of AZA on RSV in cucumber.

RESULTS.—*Time of application of AZA.*—AZA at 1 *mM* was applied at various intervals both before and after inoculation with 1:10 inoculum. The results of a series of tests indicated that preinoculation treatments were more effective than postinoculation treatments in preventing infection, and that AZA had a greater effect in suppressing starch lesions than in suppressing systemic virus symptoms (Fig. 1).

*Inoculum dilution.*—The effect of 1-*mM* AZA, applied 24 hours before inoculation with various inoculum dilutions, is shown in Fig. 2. AZA can prevent systemic virus symptoms provided the inoculum titer is not too high. Untreated plants inoculated with the same inoculum exhibited 100% systemic infection within 5 days at all these dilutions. A 5-day delay occurred in development of systemic symptoms on treated plants. Systemic symptoms of RSV on cucumber 3 weeks after inoculation were compared with normal growth on an inoculated plant treated with AZA (Fig. 7).

An inoculum of about 1:120 resulted in 50% of the treated plants exhibiting systemic virus symptoms compared with an inoculum of 1:2,250 for systemic symptoms in 50% of the untreated plants. Thus, nearly a 19-fold increase in inoculum titer is required to give an equivalent infection in plants treated with 1 *mM* AZA, indicating that about 95% of the entry or replication sites were blocked by AZA treatment.

*AZA concentration.*—AZA in concentrations of 0.03-1.0 *mM* was applied 1 day before inoculation with 1:10 inoculum to determine the effect of AZA concentration on RSV starch lesions. The results (Fig. 3) indicated a 50% inhibition of starch lesions at 0.1 *mM*. AZA produced no effect on starch lesions when included in the inoculum up to a concentration of 2

**Fig. 1-6. 1)** Effect of day of application of 1-*mM* AZA before or after inoculation with RSV on the development of local starch lesions and systemic symptoms in cucumber. Inoculations were made at 0 days. Starch lesions are given as percentage of counts on untreated cotyledons. Systemic symptoms are shown as percentage of plants expressing symptoms. Systemic symptoms were evident in 100% of the untreated plants. **2)** Effect of inoculum dilution on percentage of plants expressing RSV symptoms in cucumbers receiving 1-*mM* AZA preinoculation treatment. CK represents untreated plants at all inoculum dilutions. **3)** Effect of AZA concentration on inhibition of RSV starch lesions in cucumber cotyledons. **4)** Effect of total AZA applied as preinoculation treatment on the percentage of plants expressing RSV symptoms in cucumber. CK represents untreated plants. **5)** Effect of inoculating secondary leaves of symptomless plants that 25 days previously had received a cotyledon inoculation and a pre- or postinoculation AZA treatment. Results expressed as percentage of plants with systemic symptoms. CK represents untreated plants of same age receiving only the second inoculation. **6)** Effect of time of application of a total concentration of 2-*mM* AZA on the percentage of plants expressing symptoms of RSV in cucumber. CK represents untreated plants.

*mM*. For comparsion, similar tests were made with tobacco mosaic virus; 50% lesion inhibition occurred at 0.7-*mM* AZA, when it was applied 1 day before inoculation.

In another series of tests, AZA was applied in concentrations of 0.5-6.0 *mM* before inoculation to determine the effect on systemic virus symptoms. The 4-*mM* and 6-*mM* levels were obtained by repeated applications of 2-*mM* AZA at 24-hour intervals. All plants were inoculated with 1:10 inoculum. The percentage of plants exhibiting systemic symptoms and the time of appearance of symptoms were inverse functions of the amount of AZA applied. Data for 0.5- 2.0-, and 6.0-*mM* concentrations are shown in Fig. 4. Data for 1- and 4-*mM* concentrations, which fell in their respective positions, were omitted for the sake of figure clarity. With the higher levels of AZA, a few plants were symptomless for some time until the sudden appearance of severe symptoms in the terminal growth.

*Systemic effect.* — To determine whether AZA induced a systemic effect on the host, plants were treated with 1-*mM* AZA either 1 day before or 1 day after inoculation of the cotyledons with 1:80 inoculum. Twenty-five days after this first inoculation, systemic symptoms were present in 51% of the preinoculation-treated plants, in 80% of the postinoculation-treated plants, and in 100% of the nontreated plants. The plants showing symptoms were discarded. Cotyledons and the primary leaf were removed from the symptomless plants and the secondary leaf was inoculated with 1:10 inoculum. Untreated plants of the same age were similarly inoculated as controls. A more concentrated inoculum was used for the second inoculation because secondary leaves are less susceptible to infection than cotyledons. The results (Fig. 5) indicated that the original AZA treatment made the secondary leaves resistant to infection. The postinoculation treatment, while less effective against the first inoculation, was more effective against the second inoculation than the preinoculation treatment. A single postinoculation AZA treatment protected 20% of the plants from the first inoculation; of these, 50% remained symptomless 25 days after the second inoculation. A single preinoculation AZA treatment protected 49% of the plants from the first inoculation; of these, 32% remained symptomless 25 days after the second inoculation.

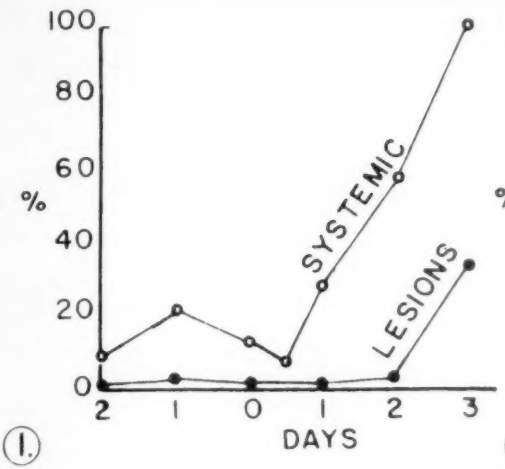
Since AZA appeared to have both a local effect in inhibiting primary replication sites and a systemic effect in inhibiting systemic virus symptoms, it was reasoned that a combined pre- and postinoculation treatment would be more effective than either treatment alone. To test this possibility, plants were treated either the day before inoculation, the day after inoculation, or both. A total concentration of 2 *mM* of AZA was applied, and the plants were inoculated with 1:20 inoculum. The results showed that the combined treatment was the most effective; 26% of the plants were protected 180 days after inoculation, compared with 10% of the plants protected by preinoculation treatment and none of the plants by postinoculation treatment (Fig. 6).

In addition to inhibiting virus symptoms completely in some plants, AZA also tended to reduce the severity of symptoms. The type of RSV culture used typically killed the primary growing point. In time, axillary buds produced new shoots which were severely mottled (Fig. 7). If the inoculum was dilute, the growing point might not be killed, but the leaves would be severely mottled. A relative score of symptom severity was obtained by rating a plant 3 for death of the growing point, 2 for a severe mottle, 1 for a mild mottle, and 0 for no symptoms. Untreated inoculated plants always had a score of 3 whereas treated plants averaged 2 or less.

*Guanine reversal.*—Guanine was only partially effective in reversing the effects of AZA. Twenty days after inoculation, untreated plants and those preinoculation-treated with guanine alone at 1 *mM* (in 1-*mM* KOH) had a symptom score of 3. Preinoculation treatment of plants with both AZA and guanine at 1 *mM* gave a score of 1.6, whereas preinoculation treatment with AZA alone gave a score of 0.9. Starch lesion counts yielded similar results. Treatment with AZA alone gave 4% as many lesions as the untreated, whereas treatment with a mixture of AZA and guanine gave 16% as many.

The systemic effect of AZA also was reversed only partially by guanine. Plants were pretreated with AZA at 1 *mM* and inoculated with 1:160 inoculum; all plants showing systemic symptoms in 14 days were discarded. Half the remaining plants were treated with guanine at 1 *mM;* the rest served as controls. Of the guanine-treated plants, 29% eventually showed systemic symptoms compared with 7% of the controls with symptoms.

Discussion.—The results indicated that AZA can completely protect some cucumber seedlings from infection by RSV. This finding is similar to that of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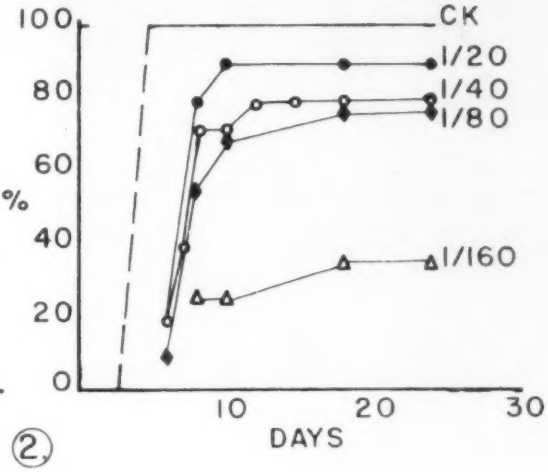
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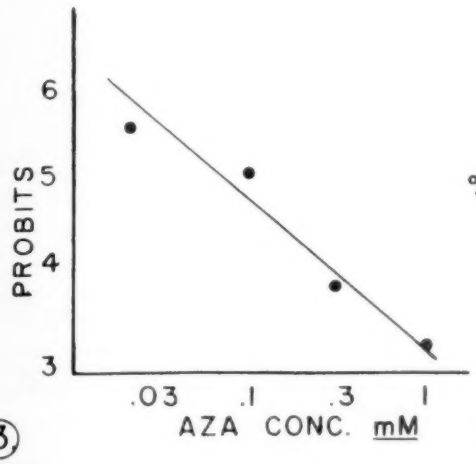
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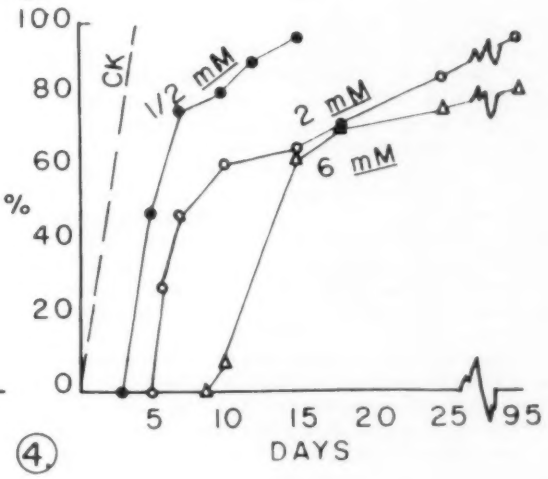
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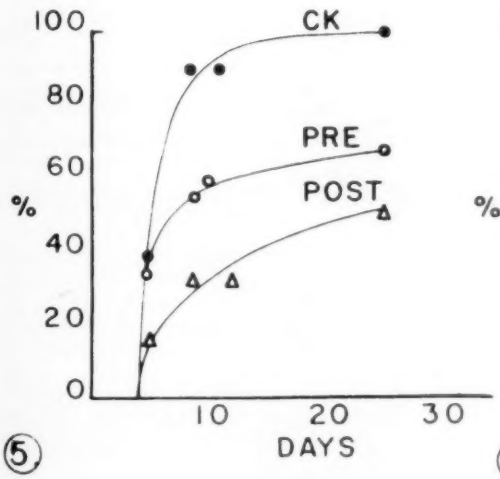
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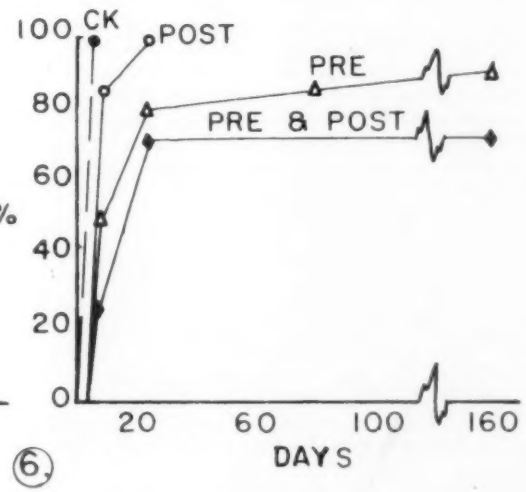
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**Fig. 7.** Systemic symptoms of RSV on cucumber 3 weeks after inoculation (left) compared with a symptomless plant treated with AZA (right).

Matthews (6, 7) with alfalfa mosaic and cucumber mosaic viruses on other hosts. The degree of protection varies directly with the amount of AZA applied and inversely with the inoculum titer. Maximum protection occurred when AZA was applied both 1 day before and 1 day after inoculation, a timing similar to maximum effect of AZA on tobacco mosaic virus infection in *Physalis floridana* Rydb. (2).

AZA apparently suppressed starch lesions more than systemic virus symptoms. In some cases, starch lesions were completely suppressed, but virus infection took place and systemic virus symptoms were evident. Possibly, AZA completely blocked the primary replication sites in the cotyledon. The virus or its translocation form may have been transported to secondary leaves which were only partially blocked by AZA, thereby allowing virus replication to take place. An alternative explanation is that some virus replication took place in the cotyledon but that the RSV was so modified by AZA that no starch lesions were formed. In time, some of the modified RSV might revert to normal RSV allowing symptom development in secondary leaves. Reversible modification of tobacco mosaic virus by AZA has been reported (2).

Since AZA had no effect on starch lesions when incorporated in the inoculum but exerted its effect when infiltrated before inoculation, it is likely that the effect of AZA is directly on the replication site rather than the entry site. This is similar to the effect of AZA on tobacco mosaic virus in *Physalis floridana* (2).

A 7-fold greater concentration of AZA was required to inhibit tobacco mosaic virus lesions in cucumber cotyledons than RSV lesions. Apparently, in cucumber, a higher rate of AZA substitution on the replication site is required to block tobacco mosaic virus replication than is required for RSV replication.

The systemic effect of AZA could be due either to a blockage of virus replication sites in the secondary growth, to a blockage of virus translocation, or both. The delay in appearance of symptoms is likely due to blockage of virus translocation; the effect of AZA in inducing milder disease symptoms could be due either to a partial blockage of replication sites resulting in a lower virus titer or to the modification of the virus by AZA.

Some treated plants were free of virus symptoms for as long as 60 days; then, the terminal developed severe symptoms. Since no control plants ever became infected, the original inoculation must have persisted somewhere in the plant as either active virus or AZA-modified virus. The sudden appearance of severe symptoms in the terminal could be due to AZA-modified virus reverting to active virus or to the AZA no longer being present in high enough concentration to block the replication sites.

Pretreatment of cotyledons with AZA was more effective than post-treatment in blocking of primary replication sites. The post-treatment, however, would seem to be more effective in the systemic effect of AZA whether on virus translocation or on secondary replication sites.

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# HOST RANGE AND PROPERTIES OF THE GLOBE ARTICHOKE CURLY DWARF VIRUS

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SUMMARY

The curly dwarf disease of globe artichoke, characterized by a twisting of shoots and dwarfing of plants, was confirmed as virus induced. Artichoke production of diseased plants was less than 25% of that of healthy plants; artichokes from infected plants were of lower quality and were produced later in the season. Inoculation of artichoke seedlings was most successful when very young plants were brushed with inoculum in 0.5% $K_2HPO_4$ and kept at 13-18°C. The host range of the virus was extended to include bachelors-button (*Centaurea cyanus* L.), China aster [*Callistephus chinensis* (L.) Nees], strawflower (*Helichrysum bracteatum* Andr.), sunflower (*Helianthus annuus* L.), and French marigold (*Tagetes* sp.). These hosts, in addition to those previously described, constitute a host range entirely within the Compositae. Zinnia was a local lesion host at low temperatures (13-18°C). The thermal inactivation range of the virus is 55-60°C; the dilution end point $10^{-3}$–$10^{-4}$, depending upon the host plant; and longevity *in vitro*, 2-3 days with zinnia sap and 1-3 hours with artichoke sap. Electron microscopic examination of sap samples from diseased artichoke, zinnia, sunflower, and bachelors-button revealed flexuous rods averaging 582 mμ long and 15 mμ wide. No such particles were found in sap from healthy plants. Anatomical studies showed a general disorganization and breakdown of tissue in and around vascular bundles of artichoke leaves showing curly dwarf symptoms. The curly dwarf virus seems distinct from any previously described virus. The artichoke latent virus was found in all field artichoke plants examined, including those infected with the curly dwarf virus.

INTRODUCTION.—What may have been the curly dwarf disease of globe artichoke (*Cynara scolymus* L.) was first observed in 1929 from the central coastal area of California. A survey of the disease in 1930 by J. B. Kendrick, Sr., and L. D. Leach (unpublished data) revealed losses of 10-40% in some fields. There were no reports of the disease from 1934-45, but since then curly dwarf has been observed in varying amounts each year in some California artichoke production areas.

Early attempts to transmit the causal agent of the disease by sap inoculation, aphid transfer, or grafting were unsuccessful. In 1950, however, Leach and Oswald (4) induced disease symptoms in artichoke, cardoon, (*Cynara cardunculus* L.), zinnia (*Zinnia elegans* Jacq.), and milk thistle [*Silybum marianum* (L.) Gaertn.] seedlings by brushing Carborundum-dusted leaves with sap from diseased artichokes. Shock symptoms of extreme leaf distortion and internal petiole necrosis appeared 25-30 days after inoculation and were followed by a dwarfing and curling of leaflets similar to the field symptoms of artichokes. For field control, the authors recommended roguing diseased plants and replanting with cuttings from disease-free areas.

Curly dwarf has been reported only from California and is 1 of 3 virus diseases found in globe artichoke. The others include a latent virus discovered by Costa, *et al.* (2), in every California artichoke field sampled, and a bright yellow leaf spot described by Gigante (3) in Sicily. In this paper, the terms "diseased" and "healthy" will refer only to the curly dwarf disease.

SYMPTOMS.—Curly dwarf is descriptive of the disease symptoms, since affected plants usually have curled leaves and are dwarfed (Fig. 1). Necrotic pockets often develop in diseased shoots, especially in the midribs; and veinal necrosis may be evident in young leaves. Internodes in all parts of the plants are shortened, causing over-all dwarfing of plants and smaller artichoke buds. Such artichokes are less desirable commercially than healthy ones and, in severe cases, become necrotic and unfit for sale.

Zinnia, cardoon, and bachelors-button (*Centaurea cyanus* L.) show stunting, systemic veinal necrosis, and twisting of leaves. Symptoms on sunflower leaves (*Helianthus annuus* L.) include chlorotic lesions sometimes ringed by necrotic margins. Strawflowers (*Helichrysum bracteatum* Andr.) have local chlorotic lesions on inoculated leaves with considerable twisting of younger, noninoculated leaves. Asters, [*Callistephus chinensis* (L.) Nees] exhibit small necrotic spots and chlorotic lesions on inoculated leaves; chlorotic vein clearing in younger noninoculated leaves; and twisting and stunting of entire plants. In general, all local symptoms on these plants appear about 2 weeks after inoculation and systemic symptoms about 3-5 weeks after inoculation. Although marigolds (*Tagetes* sp.) do not show any symptoms when inoculated with curly dwarf virus, the sap from these inoculated plants is infectious. Numerous attempts to inoculate milk thistle (*Silybum marianum* Gaertn.) were unsuccessful; however, Leach and Oswald (4) reported previously that plants become twisted and stunted when infected.

YIELD EFFECTS.—A study was made of the effect of the curly dwarf disease on artichoke production. Within an area showing relatively high disease incidence, 15 plants were selected in each of 3 categories; severe curly dwarf, doubtful curly dwarf, and apparently healthy. Subsequent indexing showed that all of the plants selected as severe curly dwarf were infected and that none of the healthy plants contained the virus. Of the doubtfuls, 8 were diseased and 7 healthy. The yield data (Table 1) show that the yield of healthy plants was about 4 times as great as that of plants

TABLE 1. Average number of marketable artichokes harvested/plant from 15 plants in each of 3 categories: severe curly dwarf, doubtful curly dwarf, and no curly dwarf

| Symptoms | Artichokes/plant/month | | | | | | Total/ plant |
|---|---|---|---|---|---|---|---|
| | Oct. | Nov. | Dec. | Jan. | Feb. | Mar. | |
| Severe curly dwarf | 0.0 | 0.2 | 1.2 | 1.6 | 1.0 | 0.5 | 4.5 |
| Doubtful curly dwarf: | | | | | | | |
| infected [a] | 1.9 | 2.4 | 1.1 | 2.0 | 0.9 | 1.9 | 10.2 |
| Not infected | 0.6 | 2.4 | 1.7 | 3.6 | 2.4 | 2.1 | 12.8 |
| Total | 1.3 | 2.4 | 1.4 | 2.8 | 1.6 | 2.0 | 11.5 |
| No curly dwarf | 3.0 | 5.3 | 5.5 | 3.4 | 1.9 | 0.7 | 19.8 |

[a] Based on reactions of plant sap on zinnia assay plants.

with severe curly dwarf. Of perhaps more importance was the tendency for the healthy plants to produce more at the early part of the season when market prices were highest. Plants showing severe curly dwarf produced an average of only 1 artichoke/5 plants in the 2-month period of October and November, whereas healthy plants yielded over 8 artichokes/plant for this same period. Moreover, the artichokes from plants with severe symptoms were usually of a lower grade than those from healthy plants.

The yields of the doubtful plants were intermediate between those of the healthy and curly dwarf plants; doubtfuls indexed as diseased produced more than the curly dwarf group; and noninfected doubtfuls yielded less than the healthy group. This might be expected, since plants with doubtful symptoms are in better condition than those obviously diseased, but in poorer condition than those considered healthy.

INOCULATION TECHNIQUES.—Optimum conditions for artificial inoculation of artichoke plants were established to facilitate further studies of the disease. Inoculation methods, additives, temperatures, and plant sizes were varied and evaluated. Diseased sap made to 0.5% with $K_2HPO_4$ was brushed on Carborundum-dusted artichoke seedlings (var. Green Globe) that had cotyledonary leaves under 5 cm in length. When these seedlings were kept at 13-18°C after inoculation, over 90% showed the characteristic symptoms of twisted leaves and veinal necrosis about 2.5-4.0 weeks after inoculation (Fig. 2). Many of these plants were transplanted to fields and later developed symptoms similar to those of naturally diseased plants.

HOST RANGE.—Ninety-four different plant species, representing 19 families, were tested for possible reaction to curly dwarf virus. All plants were inoculated when very young, in the manner found effective for artichoke seedlings, and were kept at 26°C after inoculation. In an additional inoculation of the Compositae, the plants were held at 13°C.

Infection occurred only in the Compositae and in this family only in artichoke, cardoon, and zinnia, which Leach and Oswald (4) had found susceptible; and in bachelors-button, sunflower, China aster, strawflower, and French marigold. Infection of these last

**Fig. 1.** Twisting of artichoke leaves due to the curly-dwarf virus (right). (Normal leaves at the left.) **Upper:** Seedling leaves 2.5-4.0 weeks after inoculation. **Lower:** Leaves from field-grown plants.

4 occurred only in the plants held at 13°C. No infection was observed in lettuce (*Lactuca sativa* L. var. *capitata* L. Los Angeles Market), endive (*Cichorium endivia* L.), dandelion (*Taraxacum officinale* Weber), chrysanthemum (*Chrysanthemum multifolium* Ramat.), Shasta daisy (*C. maximum* Ramond var. Alaska), cosmos (*Cosmos* sp. var. Orange Flare), calendula (*Calendula officinalis* L. var. Double Mixed), and milk thistle (*Silybum marianum* Gaertn.).

No symptoms were observed in any inoculated plants belonging to the other families tested, including the Gramineae, Liliaceae, Chenopodiaceae, Amarantha-

**Fig. 2.** Necrotic lesions on zinnia leaves infected with artichoke curly-dwarf virus about 10 days after inoculation.

ceae, Aizoaceae, Caryophyllaceae, Papaveraceae, Cruciferae, Leguminosae, Tropaeolaceae, Euphorbiaceae, Malvaceae, Umbelliferae, Verbenaceae, Solanaceae, Scrophulariaceae, Dipsaceae, and Cucurbitaceae.

ZINNIA AS AN INDICATOR.—The relatively long incubation period (2.5-4.0 weeks) and the absence of discrete symptoms are 2 disadvantages of artichoke as an indicator plant for curly dwarf virus. In zinnia, the virus had a shorter incubation period and produced more definite symptoms of leaf necrosis. Further studies were conducted with this plant. Zinnia developed small necrotic lesions on inoculated cotyledonary, first-, second-, and third true leaves 1 week after inoculation when held in a cool environment (13-18°C). More lesions resulted when the inoculum was made to 0.5% with $K_2HPO_4$. Sap samples from all of the naturally infected field artichokes and artificially infected greenhouse hosts produced such lesions when rubbed on zinnia leaves, whereas no symptoms were produced by sap from "healthy" latent virus-infected plants. Fourteen commercial varieties of zinnia were tested and all yielded similar symptoms (Fig. 2).

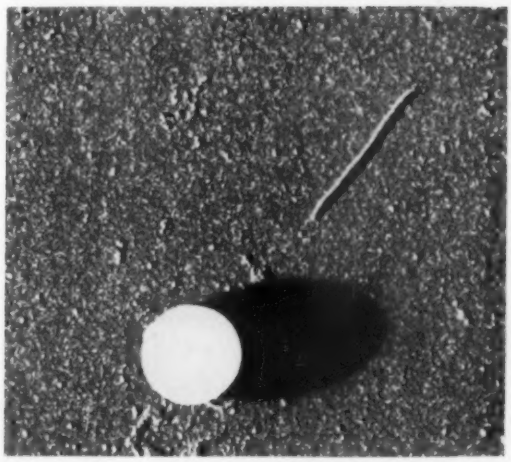

**Fig. 3.** A typical flexuous rod associated with sap from curly-dwarfed artichoke plants. Polystyrene latex sphere is 333 mμ in diameter. Similar background debris also was present in preparation from a healthy artichoke plant.

AGING IN VITRO.—Sap samples from diseased artichoke and zinnia leaves were stored as 10-ml portions in small glass test tubes at 19°C, and were used at intervals for infectivity tests. The inactivation time was 2-3 days in zinnia sap, but only 1-3 hours in artichoke sap. This shorter inactivation time is probably due to inhibitory material in the artichoke sap, as indicated by Costa, *et al.* (2).

HEAT INACTIVATION.—Tubes of sap from infected zinnias were placed for 10 minutes in a water bath at 35-60°C in 5° intervals. The thermal inactivation point of the virus was 55–60°C.

DILUTION END POINT.—The dilution end point of the virus was estimated by conventional methods, using zinnia as the test plant. Infectivity of zinnia, bachelors-button, artichoke seedling, and artichoke field plant sap was compared by number of lesions produced on zinnia leaves. The dilution end point was $10^{-3}$, except for zinnia sap, which was infectious at a dilution of $10^{-4}$. Lesion counts indicated a direct relationship between the concentration of virus in the sap and the number of lesions produced on zinnia test plants.

ELECTRON MICROSCOPY.—Samples of curly dwarf virus-infected and healthy artichoke leaves were prepared for electron microscopy by homogenizing in distilled water and diluting 1:50. Examination of these samples with an RCA model EMU-2 electron microscope showed occasional flexuous rods in sap from diseased leaves (Fig. 3). Mean length of the rods was estimated to be 582 mμ (S.D. 19.5 mμ) (Fig. 4); mean width was 15mμ (S.D. 3 mμ). Similar particles also were observed in sap of curly dwarf virus-infected zinnia, bachelors-button, and sunflower plants, the only other infected hosts examined. Although relatively few rods were found in sap of any of the plants, their concentration seemed highest in zinnia, followed by artichoke, sunflower, and bachelors-button. No such

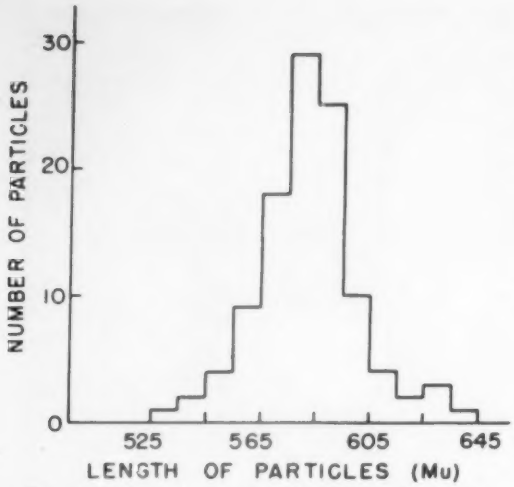


**Fig. 4.** Histogram of particle lengths found in preparations made from curly-dwarfed artichoke plants.

rods were seen in healthy, seed-grown members of these species, or in artichoke plants infected only with the latent virus.

PURIFICATION.—Attempts to purify the artichoke curly dwarf virus by the usual low- and high-speed centrifugation methods were unsuccessful. The particles, however, did accumulate in a specific region following centrifugation in a sucrose density-gradient system (1), but their concentration was so low that no real purification was obtained.

ANATOMICAL STUDIES.—Field artichoke plants showing typical disease symptoms and inoculated artichoke seedlings with severely twisted leaves were examined and compared with healthy plants of comparable age and size. Tissue was fixed in Karpechenko's solution, imbedded in paraffin, sectioned, and stained with iron-alum hematoxylin (5). No inclusion bodies were found in the cells of diseased plants. The only marked abnormality observed in diseased plants occurred in and around vascular bundles, especially in areas where necrosis had developed. Such tissues showed extensive disorganization and contained intercellular cavities. The over-all effect was a deterioration of vascular bundles including xylem, phloem, and parenchyma tissues.

DISCUSSION.—Studies of the curly dwarf virus are complicated by the presence of the artichoke latent virus which apparently infects all field artichoke plants. Possibly, curly dwarf symptoms are the result of the 2 viruses acting together. Attempts to separate the viruses, so that comparisons could be made between the symptoms caused by the curly dwarf virus alone and in association with the latent virus, were unsuccessful. Perhaps, more than 1 latent virus is present in artichoke plantings, since plants are vegetatively propagated and any virus once introduced would be readily maintained in later plantings.

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## PHYTOPATHOLOGICAL NOTES

### PHYTOPHTHORA PARASITICA, THE CAUSE OF ROOT ROT, CANKER, AND BLIGHT OF BOXWOOD

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Contribution from Plant Pathology, North Carolina Agricultural Experiment Station, Raleigh; published with the approval of the Director of Research as Paper No. 1279 of the Journal Series.

A disease of boxwood (*Buxus sempervirens* L.) was encountered frequently in North Carolina during 1959 and 1960. A similar disease has been referred to as "wilt and canker" and has occurred for many years in the middle-Atlantic and southeastern United States. Weiss and St. George (2) recognized wilt and canker as different symptoms of the same disease syndrome

TABLE 1. Results of inoculations of English boxwood roots with *Phytophthora parasitica* and *Meloidogyne javanica*

| Inoculum | | No. of nematode larvae recovered from 30 roots, 1 month after inoculation | No. of plants yielding *P. parasitica* 30 days after inoculation/no. inoculated | No. of plants with blight symptoms | Range in days for development of blight symptoms |
|---|---|---|---|---|---|
| *P. parasitica* | *M. javanica* | | | | |
| PE-1 | + | 28 | 3/3 | 0 | |
| | — | 0 | 3/3 | 0 | |
| 1A-8 | + | 10 | 3/3 | 1 | 54 |
| | — | 11[a] | 3/3 | 2 | 51-60 |
| 3A-23 | + | 0 | 3/3 | 3 | 51-88 |
| | — | 0 | 3/3 | 1 | 50 |
| None | + | 112 | 0/3 | 0 | |
| | — | 0 | 0/3 | 0 | |

[a] Unexplained contamination.

and suggested that these symptoms result from freezing injury. Although *Volutella buxi* (D.C. ex Fr.) Berk. & Br. and *Verticillium buxi* (Lk.) Auers. & Fleischhack were characteristically associated with the disease, they concluded that these organisms are not the primary parasites but are secondary invaders affecting boxwood after it has been injured by freezing.

Although Andrus (1) isolated *Phytophthora parasitica* Dastur from boxwood in Maryland in 1933 and suggested its potential importance as a pathogen, he did not associate the fungus with any disease of boxwood. In the past 2 years, *P. parasitica* has been isolated consistently from plants having canker and blight symptoms. The objective of this investigation was to determine the relationship of *P. parasitica* to root rot, stem canker, and blight of boxwood.

METHODS.—Isolations were made by inserting tissues from infected plants into apples and plating subsequently infected apple tissue on water agar. Inoculum was grown on sterilized oats at 28°C for at least 10 days or on corn-meal or oatmeal agar in petri dishes at room temperature for 7 days.

Most inoculations were made by adding the fungus, grown on sterile oat grains, to the rhizosphere of potted boxwood plants or to pasteurized soil subsequently used for potting rooted cuttings of boxwood. In some inoculations, 1 $cm^2$ of mycelium from oatmeal or corn-meal agar was applied to wounded or nonwounded stem surfaces of healthy boxwood plants and held in place with moistened, sterile cotton wrapped with cellophane. Noninoculated plants served as controls. Plants were kept in the greenhouse with night temperatures of 24–27°C and day temperatures of 31–38°C. Additional tests included comparisons of 3 isolates of *P. parasitica* with and without the root-knot nematode *Meloidogyne javanica* (Treub) Chitwood. The nematode inoculum consisted of a mixture of infested soil and infected tomato roots. Approximately 200 g of inoculum were added to each of twelve 10-in. pots containing a boxwood plant.

RESULTS.—*P. parasitica* inoculated in wounded and nonwounded stems of English and American varieties of boxwood produced infections in 7–10 days and stem cankers after approximately 1 month; blight symptoms appeared 30–90 days after inoculation. *P. parasitica* was reisolated from 1 of 6 infected plants 45 days after inoculation, the other plants yielded 1 or more species of *Fusarium*, *Trichoderma*, *Penicillium*, or *Gliocladium*. Control plants remained healthy.

Studies on the interaction of *P. parasitica* and *M. javanica* indicated that *M. javanica* did not increase disease severity. Although substantial numbers of root-knot larvae colonized the roots of boxwood plants not inoculated with *P. parasitica*, colonization was inhibited in the presence of the fungus. Since *P. parasitica* caused a severe root rot, healthy root tissue essential for the development of the nematodes was limited. Three months after inoculation, 7 of 18 plants inoculated with *P. parasitica* showed canker and blight symptoms. The time required for the appearance of blight symptoms was 50–88 days (Table 1); no record was made of the appearance of canker symptoms. Wounds in stems or roots were not necessary for infection since there was no significant difference in numbers of infected plants, or in infection rate, with or without mechanical injuries to stems or injuries made by nematodes in the roots. One isolate of *P. parasitica* induced stem cankers and blight when inoculated in stems of healthy boxwood, but failed to induce these symptoms after 5 months in inoculated roots of similar plants. By contrast, other isolates of *P. parasitica* induced canker and blight when inoculated in roots; stem inoculations were not attempted with these latter isolates. Root rot was more severe with the latter isolates.

Five isolates of *P. parasitica* were tested for pathogenicity to rooted cuttings of American and Japanese boxwood. Root rot developed rapidly and 17 days after inoculation *P. parasitica* was recovered from 14 of 18 inoculated plants. Each of the 5 isolates was recovered 1 or more times. *P. parasitica* was not recovered from 3 control plants.

SYMPTOMS.—The first obvious symptoms of the disease are loss of luster and a simultaneous color change in the foliage from dark to light green. In quick succession, the leaf is inclined upward and the lateral margins roll inward as a result of desiccation. Finally, the leaf is bleached to a light straw color. One or more branches, or the whole plant, may develop these symptoms. The bark at the base of affected

branches dies and with desiccation it separates from the wood and may be removed easily. Frequently, a thin layer of dead tissue appears in the cambial region. After 1 month or more, the xylem usually becomes superficially darkened. Root rot precedes the development of symptoms in foliage and branches.

DISCUSSION.—Boxwood plants infected with *P. parasitica* often develop symptoms that are indistinguishable from symptoms of the "wilt and canker" disease of boxwood which is considered caused by freezing injury of unhardened wood or unseasonably active cambium. Similarities of the 2 disorders are striking. Stem cankers are present in either case and isolations from diseased tissues, using conventional laboratory media, usually yield *Verticillium buxi* and *Volutella buxi;* species of *Fusarium*, *Penicillium*, and *Trichoderma* also may be recovered. The sudden desiccation of 1 or more branches, or an entire small plant, resulting in the changing of foliage color from dark green to a pale, straw color is apparently common to both diseases. Whether or not root deterioration is a concomitant of freezing injury is not recorded. Root rot is a symptom, however, of the disease caused by *P. parasitica* and indeed precedes symptoms associated with desiccation of branches and foliage.

It is probable these 2 boxwood disorders exist independently of each other. It is equally probable that, heretofore, the disease caused by *P. parasitica* has been confused with the disorder caused by freezing injury.

The name, *Phytophthora* blight, is proposed for the disease caused by *P. parasitica* thus differentiating it from the similar disorder of boxwood attributable to freezing and referred to as "wilt and canker."

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## EVIDENCE OF GENE POOLS FOR PATHOGENICITY IN SPECIES OF HELMINTHOSPORIUM

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Many species of phytopathogenic fungi are composed of physiological races or biotypes distinguished by qualitative or quantitative differences in reaction of varieties or selections of host species. This demonstrates that the pathogenicity of such species to specific hosts is conditioned by a number of genes. Presumably, the complexity of the genetic mechanisms for pathogenicity is proportional to the degree of specialization, although the extent to which specialization can be determined is influenced by the availability of appropriate differential varieties. Fungus pathogens possess complex mechanisms for pathogenicity to given plant species, *i.e.*, crosses between biotypes or strains often produce hybrid progenies that differ markedly in pathogenicity from parental biotypes. It is not known, however, whether species exhibiting specialization possess genes for pathogenicity other than those required to attack a given plant species.

Further, many plant pathogenic fungi attack a wide variety of plant species and often the host range includes species in many plant families. It is not known whether pathogens with wide host ranges have genes specific for pathogenicity to each host or certain genes which condition pathogenicity to more than 1 host or 1 set of genes which govern pathogenicity to all hosts.

Recent studies have demonstrated cross-fertility between graminicolous *Cochliobolus* spp. having *Helminthosporium* conidial stages and between *Cochliobolus* spp. and *Helminthosporium* spp. with no known sexual stage (1). Concurrent studies on the pathogenicity of these and related *Helminthosporium* spp. to species in 22 genera of the Gramineae have shown that pathogenic *Helminthosporium* spp. are not host specific, but attack a wide variety of gramineous species (2). Hybrid progenies from certain interspecific crosses have been studied on differential plant species selected on the basis of their reaction to the parental isolates, including species resistant to neither, 1, or both of the parental strains.

Hybrid progenies from crosses of isolates of *C. carbonum* Nelson (*H. carbonum* Ullstrup) × *C. victoriae* Nelson (*H. victoriae* Meehan & Murphy) frequently segregate 1:1:1:1; ½ pathogenic to corn or oats, ¼ pathogenic to both hosts, and ¼ nonpathogenic to both parental hosts. The last 2 classes are considered recombinant classes. Selected data obtained from 4 different crosses of *C. carbonum* × *C. victoriae* are summarized herein. One isolate of *C. carbonum* was used in crosses with 4 isolates of *C. victoriae*.

The 5 parental isolates and all parental and recombinant ascospore isolates thus far examined are pathogenic to certain common hosts, including *Sorghum vulgare* var. *sudanense* (Piper) Hitchc. (Sudan grass), *Pennisetum glaucum* (L.) R. Br. (pearl millet), and *Lolium multiflorum* Lam. (Italian ryegrass). Hybrid ascospore isolates nonpathogenic to both parental hosts are pathogenic to other gramineous species, indicating that the genes in *C. carbonum* for pathogenicity to corn and the genes in *C. victoriae* for pathogenicity to oats are not required, at least in their original arrange-

ment, for pathogenicity to other hosts. Further, both parental species apparently possess additional genes for pathogenicity and these genes function independently from the genes for pathogenicity to corn and oats. The similar behavior of hybrid progeny representative of both recombinant classes supports this concept.

All the parental isolates were nonpathogenic to *Cynodon dactylon* (L.) Pers. (Bermuda grass) in repeated inoculations. Certain hybrid ascospore isolates, some pathogenic to both corn and oats and others nonpathogenic to both parental hosts, were highly pathogenic to Bermuda grass, inciting well-defined leaf lesions. These results indicate that recombinations occurred between genes not directly a part of the gene mechanisms required for pathogenicity to corn and oats.

Although approximately 50% of the hybrid ascospore isolates were similar to one or the other parental isolate in their pathogenicity to corn or oats, they exhibited differences in pathogenicity to other hosts. Certain hybrid isolates pathogenic to oats were nonpathogenic to *Bromus catharticus* Vahl (rescuegrass) and *Hordeum vulgare* L. (barley), species susceptible to *C. victoriae*. Likewise, certain progeny pathogenic to the corn inbred line Pr, susceptible to race 1 of *C. carbonum*, were nonpathogenic to *Sorghum vulgare* Pers. (sorghum), a species susceptible to *C. carbonum*. Although the genes for pathogenicity to corn and oats apparently segregated independently, the behavior of these hybrid isolates suggests that recombinations occurred between other genes for pathogenicity in the 2 species.

It is likely that additional variation in pathogenic potential will be observed as hybrid progenies of other interspecific crosses are studied. One of the objectives of future investigations will be to determine whether gene pools for pathogenicity are composed of 1 or more gene systems which function independently of other gene systems. Possibly, a gene system for pathogenicity is composed of 1 or more major genes and additional minor genes. Studies on the inheritance of pathogenicity in *C. carbonum* have indicated that race 1 and race 2 are differentiated by alternate states of a single gene (3). All attempts to demonstrate transgressive inheritance have been unsuccessful. Differences in pathogenicity to other gramineous species, however, are evident when isolates of race 1 and 2 are compared. Studies on the pathogenic potential of ascospore isolates from crosses of race 1 × race 1, race 2 × race 2, and race 1 × race 2 should provide information on the complexity of the gene pool in *C. carbonum;* and could provide data on the presence or absence of linkage groups within and between gene systems.

Apparently, these studies present the initial evidence that plant pathogenic fungi possess gene pools that include not only genes for pathogenicity to specific hosts, but also additional genes for pathogenicity to other hosts. Hybridization between pathogenic species provides the vehicle for recombination between genes present in each species. The extent to which such recombinations occur in nature is difficult to assess. Selection pressures imposed upon pathogens, as a result of the incorporation of added genes for resistance into varieties of crop plants or the introduction of new species of plants, may influence the extent and frequency of recombination within these gene pools.

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# NEWS

*Editor:* K. W. KREITLOW, Plant Industry Station, Beltsville, Md.

**Plant pathologist** Russell A. Hyre, Crops Protection Research Branch, U.S.D.A., was transferred August 31, 1961, from Newark, Delaware, to University Park, Pennsylvania, to continue his epidemiological research on late blight of potatoes and tomatoes and certain other selective diseases. At University Park, Dr. Hyre will have excellent growth control chambers and other laboratory facilities for conducting this research. He will also have an opportunity to cooperate with plant pathologists of the Pennsylvania Agricultural Experiment Station who have considerable interest in this area of research.

**Nematologist** R. S. Pitcher, East Malling Research Station, Maidstone, Kent, England, was the recipient of a recent grant from The Rockefeller Foundation to provide support which will enable him to confer with nematologists in the U.S.A. during November and December. He will spend several days with nematologists located in California, Arkansas, and Florida prior to presenting an invitational paper in the Nematology Symposium at the annual meeting of the American Phytopathological Society at Biloxi, Mississippi.

**Plant pathologist** Harry C. Young, Jr., Botany and Plant Pathology Department, Oklahoma State University, Stillwater, has been granted a one year leave of absence. He will pursue the following research: A study of the role of the alternate hosts, Thalictrum and Isopyrum species, in the variability of the wheat leaf rust fungus, *Puccinia recondita*, in nature. Field studies and plant collections will be made in New Mexico, Colorado, and Wyoming, and greenhouse and laboratory studies will be made at the University of Minnesota, St. Paul. These studies are being supported by grants from the John Simon Guggenheim Foundation and the Rockefeller Foundation.

**Grass rust fungi** of the Mexican border area will be studied by George B. Cummins of the Department of Botany and Plant Pathology, Purdue University, under a National Science Foundation grant. Dr. Cummins will be on sabbatical leave from July 1, 1961, to June 30, 1962.

**Forest pathologist** John Ohman, a recent graduate in plant pathology and botany at the University of Minnesota, has been employed by the U.S.D.A. Forest Service, Lake States Experiment Station, St. Paul, Minnesota, where he will conduct studies on heart rot of northern hardwoods.

**Phytopathologist** Thomas Theis was recently appointed Chief of the Tobacco and Sugar Crops Research Branch, U.S.D.A., Beltsville, Maryland, as successor to Dr. H. M. Tysdal. Dr. Theis was formerly Assistant Officer in Charge of the Federal Experiment Station at Mayaguez, Puerto Rico.

**Pathologists** B. G. Chitwood and R. B. Hine recently joined the Department of Plant Pathology, University of Hawaii. Dr. Chitwood will investigate nematode diseases of economic plants in Hawaii, and Dr. Hine will study primarily soil-borne fungus diseases.

**A long-out-of-print** book on Phytopathological and Botanical Research Methods by T. E. Rawlins has been reproduced in both paper cover and library binding by University Films, Inc., 313 N. First Street, Ann Arbor, Michigan.

**Pathologists** D. E. Munnecke and R. M. Endo, formerly located at the University of California, Los Angeles, have transferred to the Department of Plant Pathology, Riverside, California. Dr. Munnecke will continue his research on the fate of fungicides in soil and on diseases of vegetatively propagated ornamental plants; Dr. Endo will continue to work on diseases of turf grass and woody ornamental plants.

**Pathologist** L. C. Knorr returned August 18 to his post at the Citrus Experiment Station, University of Florida, Lake Alfred, after serving one year in Egypt as Agricultural Officer for the Food and Agriculture Organization of the United Nations. In collaboration with Dr. Farid Nour-Eldin of the Ministry of Agriculture of the United Arab Republic, Dr. Knorr investigated the virus diseases affecting citrus. Other citrus-growing areas visited by Dr. Knorr were those in the Aden Protectorate, Palestine, Sicily, and Morocco.

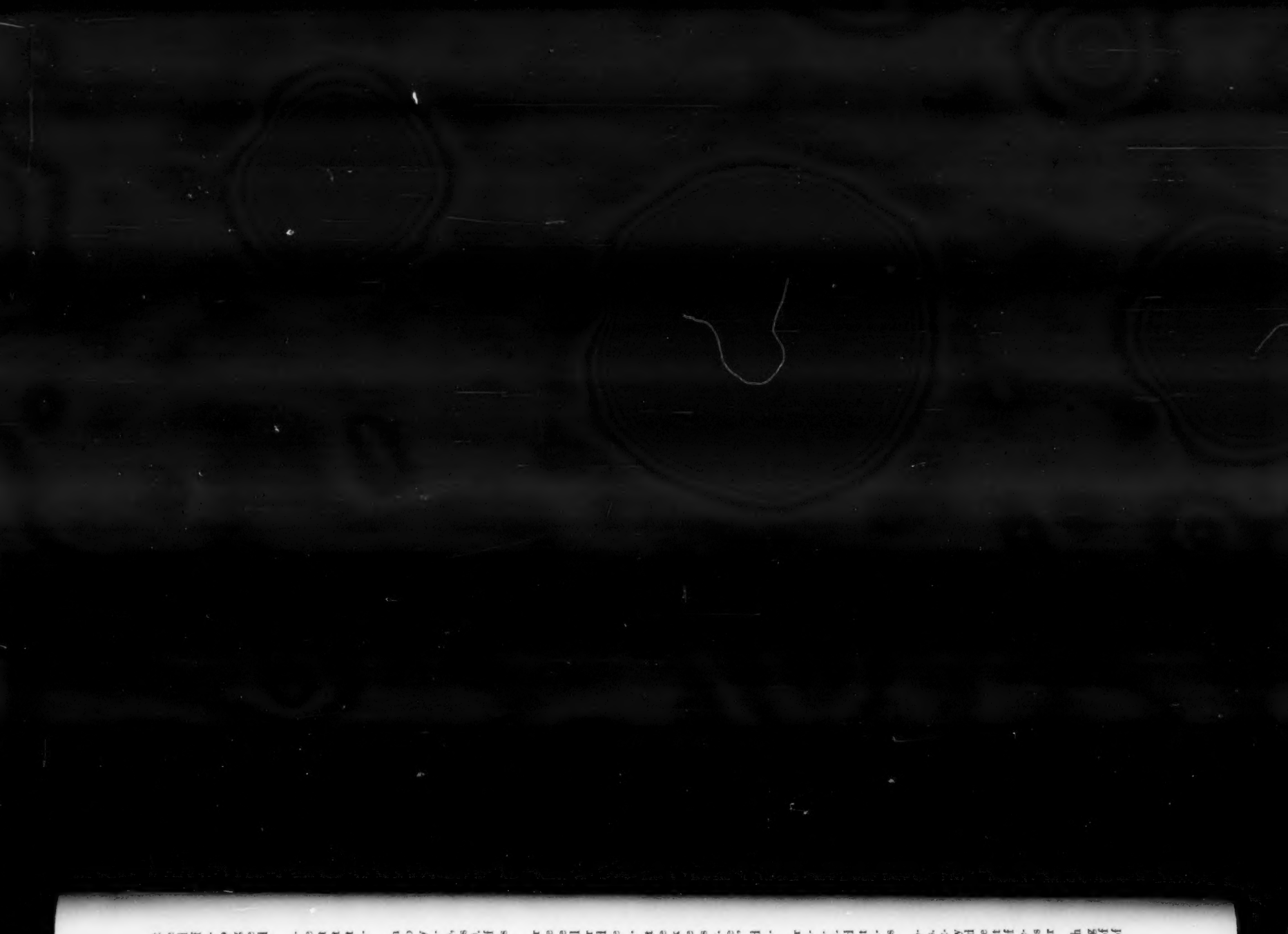
**Plant pathologist** Robert W. Toler, Vegetables and Ornamental Research Branch, U.S.D.A., joined the staff of the Plant Pathology Department, Coastal Plain Experiment Station, Tifton, Georgia, July, 1961. Dr. Toler is a recent graduate of North Carolina State College. He will conduct investigations of southern pea diseases with initial emphasis on virus disease problems in Georgia.

**A prize** of I.E. 500 (approximately $1000.00) has been awarded by the Government of the United Arab Republic to phytopathologist Farid Nour-Eldin of the Egyptian Ministry of Agriculture for outstanding contributions to the country's agriculture. Of 87 scientists submitting 560 papers, Dr. Nour-Eldin was chosen one of 16 winners on the basis of the best papers published during the period 1958-1960. Dr. Nour-Eldin is Head of the Virology Department of the Section of Plant Pathology and a specialist in viruses affecting citrus.

**Pathologists** Lawrence R. Schreiber and David E. Zimmer have recently received their Ph.D. degrees from Purdue University. Dr. Zimmer has accepted a position with the U.S.D.A. at Utah State University, Logan, where he will conduct research on diseases of safflower. Dr. Schreiber has accepted a position with the U.S.D.A., Shade Tree and Ornamentals Plant Laboratory, Delaware, Ohio, where he will be working on vascular diseases of woody ornamentals.

**Deaths**—Dr. J. E. Livingston, 52, Head of the Department of Botany and Plant Pathology at the Pennsylvania State University, died unexpectedly August 15, of a heart attack while attending a meeting in Washington, D. C. A Jesse Livingston Memorial Lectureship and Library Fund has been established by the Department of Plant Pathology. Anyone interested in contributing to the Fund, please make checks payable to "The Jesse Livingston Memorial Fund" and send to Dr. J. E. Wright, Department of Botany and Plant Pathology, The Pennsylvania State University, University Park, Pennsylvania.

Dr. Gotthold Steiner, 75, former Chief Nematologist for the U.S.D.A., died of a heart attack August 21 while visiting in Maryland. A native of Berne, Switzerland, Dr. Steiner came to the United States in 1921 to study at Yale University. In 1923 he joined the Agriculture Department and carried on much of his pioneering nematode research at Beltsville, Maryland. Dr. Steiner retired from the Agriculture Department in 1955. At the time of his death he was Nematologist, University of Puerto Rico, Rio Piedras.

Dr. Sears P. Doolittle, 71, retired Principal Plant Pathologist of the U.S.D.A., died August 9 after a brief illness. Dr. Doolittle, who had been with the Agriculture Department since 1918, retired in March 1960. He was widely known for his research investigations on the nature and control of diseases, particularly virus diseases, in vegetable crops. Dr. Doolittle was a member of a research team that received the Superior Service Award for the discovery of an alkaloid in certain tomato plants which accounts for resistance to wilt disease. He also had been cited for his competence as a consultant on plant diseases and their control.

Professor Russell H. Larson, 56, long identified with Wisconsin's potato industry, died August 29 after a long illness. During his professional career at the University of Wisconsin he published more than 80 papers on diseases of potato, onion, and cabbage.